Integrin signaling at 2hr and 48hr post-eccentric exercise in heat treated rat skeletal muscle

Zachary Aaron Graham

Committee Members

Phil Gallagher, PhD (Advisor)

Chad Touchberry, PhD

Phill Vardiman, PhD

List of Abbreviations

EE=ccentric exercise

ERK1/2=Extracellular-signal regulated protein kinase

FAK=focal adhesion kinase

HS=heat shock

Hsp= heat shock protein

IN=integrin

MT=mechanotransduction

SH=src homology

SM=skeletal muscle

SOL=soleus

SS=shear stress

Tyr=tyrosine

VLR=vastus lateralis red

VLW=vastus lateralis white

WT=wild type

Abstract

Introduction: Integrins (IN) are heterodimers made up of an alpha subunit and a beta subunit. They are transmembrane proteins that are capable of detecting and relaying signals from the extracellular matrix to the cytosol and vice versa. IN have been implicated in mechanotransduction and shear stress by becoming activated by these forces and starting intracellular cascades. Heat shock proteins are a highly conserved group of proteins that help protect cellular function by chaperoning and refolding proteins and by maintain actin structures of the cellular membrane. **Methods:** Heat shocked (HS+EE) and non-heat shocked (EE) male Wistar rats performed a two hour downhill treadmill exercise. The soleus (SOL), vastus lateralis white (VLW) and red (VLR) were harvested at 2hr and 48hr post exercise and tested for IN beta1 subunit, total and deactived c-Src527 and total and active ERK1/2 via SDS-PAGE and Westerm immunoblotting. **Results:** There were no significant differences between the treatment groups (EE and HS+EE) as well as the treatment groups versus controls in all muscles and at both time points. **Conclusion:** A two hour downhill treadmill protocol is not sufficient enough to activate IN and downstream proteins in healthy HS+EE and EE rat skeletal muscle at 2hr and 48hr post-exercise.

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Chapter 1

Integrin Signaling

The forces faced by skeletal muscle during an eccentric exercise protocol are generally too much to maintain cellular integrity. This can cause significant trauma to the muscle, inciting cellular reconfiguration and inflammation. How the muscle cell protects itself during these high stress events is not clearly understood. One possibility is cellular mechanotransduction, the stretching of the cellular membrane, which may activate a membrane protein and start a signaling cascade aimed at cell survival. Integrins are a family of proteins located on the membrane that have been shown to sense mechanotrandsuction (10) and could be a viable communicator in damaged muscle cells.

Integrins are necessary proteins that span the length of the cell membrane (39). They have the unique ability to receive signals from outside the cell and relay that information to various proteins inside the cell (50). The role of integrins has mainly been looked at in pathological conditions such as cancer (96) and muscular dystrophy (12) as well as cells where stress is constant, such as endothelial (105) and cardiac cells (85), but not a lot is known within healthy skeletal muscle. Integrins respond to cytoskeletal stress and shear stress by becoming conformationally changing their structure, activating proteins in the cytoplasm (15).

A protein responsible for potential cell survival via integrin signaling is focal adhesion kinase (FAK). FAK is located around focal adhesions, the area where integrins interact with the extracellular matrix. This could allow for quick signaling from integrins when a stress may be present. FAK is also responsible for activating two signaling pathways

which serve to maintain cell survival: the ERK1/2 pathway mediated by c-Src (17) and the PI3K pathway (18).

Another set of proteins that protect the cell are heat shock proteins (Hsp). These proteins respond to cell stress and protect the cell by chaperoning proteins and refolding damaged or misfolded ones (36, 110). It has also been shown that a specific type of Hsp, Hsp27, can activate FAK (56), perhaps causing another way to combat the cell during stress. These proteins respond quickly in an effort to maintain homeostasis and have been found to be upregulated with eccentric exercise (38).

Theoretical Basis

The basis behind this study is trying to understand how integrins affect skeletal muscle cells during a bout of eccentric exercise in rats. By examining how integrin signaling affects intracellular pathways in cancer and endothelial cells it is possible to extrapolate and predict what may happen in heat shocked skeletal muscle and non-heat shocked muscle after eccentric exercise. The forces that get transmitted along the membrane of a skeletal muscle cell will be detected through integrins and the cell will accommodate those signals for increased chances of cell recovery and survival. Heat shocking before the exercise, should provide even more insurance for cell stability and survival.

Statement of the Problem

Currently, there is no study that has looked at integrin signaling in heat shocked skeletal muscle prior to a bout of eccentric muscle damage. The purpose of this study is to compare integrin recruitment and FAK signaling following exercise induced muscle damage in heat-treated versus non heat-treated rats.

Hypothesis and Specific Aims

Hypothesis

The hypothesis of the study is that eccentrically exercised rats will have an increase in integrin signaling versus controls and the heat shocked proteins will augment intracellular protein levels responsible for cell survival.

Specific Aim #1

Determine if heat shocked and control rats that have been eccentrically exercised have changes in expression of the muscle specific integrin alpha7beta1 via densiometry of total beta1 subunit; total, phosphorylated and the ratio of phospho to total ERK1/2; and total, phosphorylated and the ratio of phospo to total c-Src in the soleus (S), vastus lateralis red (VLR) and vastus lateralis white (VLW). Phosphorylated c-Src will be looked at tyr site 527 and a decrease in content will be a sign of activity in the cell.

Rationale

The rationale for doing this study is to increase the body of literature regarding the role of integrins and skeletal muscle. Integrins have been studied heavily in pathological conditions but not as much in response to exercise in muscle. For example, in Duchenne's muscular dystrophy, integrin levels are staggeringly high as the cells try to maintain their ability to sustain themselves after the force of a contraction. Trying to treat muscular dystrophy with Hsp has been hypothesized because of the role the protein takes in the cell. This makes sense in that Hsp have thought to be a way help muscle recover cell function in dystrophin lacking cells and other myopathies. In healthy

skeletal muscle, if integrin levels increase during exercise it may provide another mechanism to protect the cell and return to homeostasis quicker.

In cancer cells, mediating proteins through integrins is considered harmful, as it keeps the cells alive and growing. However, in skeletal muscle this is beneficial. The downstream proteins have been studied extensively and will show further proof of the role of integrins in skeletal muscle sustainability and survival rates.

Chapter 2

Review of Literature

Introduction

Integrins (IN) are a family of transmembrane proteins that help stabilize the cell while also taking cues from the extracellular matrix to communicate with the interior of the cell. This activates varying cascades and intracellular pathways (70). In mammals, IN are composed of 8 beta subunits and 18 alpha subunits that can come together to form a set number of 24 integrins (39). These combined subunits are then capable of detecting extracellular activity to direct intracellular communication and vice versa (39). They are also responsible for cell-to-cell interactions during muscle maturation and in diseases related to skeletal muscle, such as muscular dystrophies (67).

While the number of publications in the literature concerning IN and mammals is extraordinary (typing 'integrins mammals' in PubMed listed 35,822 hits as of this writing), this review will concern itself with the diversity of actions in regards to proteins downstream of IN signaling and the diversity of actions that aspects of a particular protein can have (c-Src, in particular). All of this will be used to help extrapolate on how fluid or shear stress (SS) and mechanotransduction (MT), which are constant forces for cells like vascular endothelial cells, could possibly relate to eccentric exercise (EE) and how this may alter certain activities of intracellular proteins in skeletal muscle (SM). It needs be noted, however, no studies can be located regarding alterations of IN signaling by SS in SM. There have been few studies published regarding IN and MT and alterations to their downstream changes concerning mature SM cells. However, there are

a litany of studies regarding changes to SS and MT with IN signaling in endothelial vascular cells, cancer cells, osteoblasts, cardiac myocytes and so on. With that being stated, there must be an underlying assumption that similar mechanisms could happen in regards to mature SM, especially considering these functions in cardiac cells. It is understood that it may not be the case but it is necessary for the context of this paper.

Furthermore, this review will highlight how IN pathways may affect four distinct proteins related to SM signaling and function: focal adhesion kinase (FAK), c-Src, phosphoinositide 3-kinase (PI3K) and its downstream proteins, along with the mitogen activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2). After the independent roles and characteristics of these proteins have been briefly described there will be a section that deals with relationships each may have with the other and brief extrapolations regarding eccentric exercise. The last section of the paper will be dedicated to a brief discussion of heat shock proteins (HSPs) and how specific ones, Hsp25/27 and Hsp70/72, react to EE.

MECHANOTRANSDUCTION

MT is the ability of the cell to detect a physical act upon the cytoskeletal membrane and transmit it into an intracellular signal. These forces can be detected from stretching (similar to muscle contractions) or SS (blood flowing through vascular endothelial cells, for example) in a large variety of cells. In SM, p70S6K is a protein upregulated during exercise that ultimately increases protein synthesis, cellular hypertrophy and cell survival. Baar and Esser used electrical stimulation to cause high-resistance muscle contractions in the plantarflexors to cause an eccentric load to the dorsiflexors (tibialis anterior (TA) and

extensor digitorum longus (EDL)) (3). Twice a week the muscles were contracted at a rate of 60 times per 20 minutes, with the protocol lasting 2 months. In TA they saw a 14.3% increase in muscle size with an increase of 13.9% in EDL. They also saw a positive correlation with muscle mass and change in p70S6K (r=.998), demonstrating that stretch-based MT mediated by electically-stimulated contraction increased protein synthesis in rat SM. In conditions such as Duchenne's muscular dystrophy, MT is hindered in the muscle cell because dystrophin is not there to dissipate the force through the cell membrane. In a Duchenne's muscular dystrophy mouse analog with increased Akt expression, another protein responsible for cell growth and survival, there was an increase in eccentric force prevention and a vast increase of genes related to z-disks and constameres (8). Contractions were made *in vivo* by using an electronic stimulator to produce eccentric forces over a course of three weeks. After this training set, there was only a 14.8% drop in force resistance over a set of eccentric contractions compared to controls, which lost 34.8%. This shows that repeated bouts of MT in SM can alter the biochemical processes inside the cell, increasing protein content and cell size, all in a manner to protect the cell.

INTEGRIN ALPHA7BETA1

Regulation with Damage

There have been limited studies regarding IN signaling in mature SM. The principal IN in SM is alpha7beta1. The alpha subunit determines the outside signal through different ligand detections and the beta subunit is connected to actin via different protein complexes (10). IN connect myofibers to the ECM and they are heavily located around

myotendenous junctions (MTJ) (44). In a study to find the necessity of each subunit, researchers noted a decrease in the alpha7 IN gene, *ITGA7*, in patients with noted congenital myopathy. By using three case studies and RT-PCR, they were able to show that these decreases are caused by two different base pair mutations in the intron for two of them and a substitution of another base pair that caused a premature stop codon in the other case study (34). This indicates that coding for alpha7 in necessary for normal muscle functions. It also appears that this IN aids in muscle recovery. Sprague-Dawley rats that had their soleus transversely cut to create damage followed by suturing the fascia, allowing the injured muscle to heal. The alpha subunit was upregulated at days 3, 10 and 28 afterwhich they returned to basal levels. The beta subunit was present in all time courses but ablated during days 3 and 10 (44). This shows that during healing and regenerations, alpha7 plays a large role in formation and recovery but at rest, beta1 is responsible for the function of the complex. Using wild type (WT) and alpha7 null mice and exposing them to a downhill treadmill protocol, the researchers were able to determing if there was any integrin input to help with recovery. There was a 5.4 fold increase in alpha7 *mRNA* in WT versus animals that did not exercise 3 hours after, with a return to baseline at 24 hours. There was also an increase in alpha7 concentration at the MTJ immediately post exercise and 24 hours later (11). Furthermore, a study comparing used WT and mice with varying increased alpha7BX2 isoforms (the most common in adult SM) that were exposed to a downhill treadmill protocol. It was found that there was an increase in MAPK activity, specifically c-JNK, p38, and ERK1/2 in WT. However, in the mice overexpressing the alpha7BX2 isoform, there was no change from pre to post exercise, meaning that IN signaling could be a negative regulator of MAPK.

This study also showed that the overexpressing mice attenuated all signals through the PI3K pathway, especially p70S6K (10), indicating that IN may inhibit cell growth and cell survival by shutting off the PI3K pathway.

FAK

FAK and Integrins

Focal adheision kinase (FAK) is a 125 kDa non-receptor tyrosine kinase that is located in the cytoplasm (46). X-ray chrystallography has shown that FAK contains four main regions: an N-terminal FERM (band 4.1, ezrin, radixin, moesin homology), a linker region that contains the main phosphorylation site, an active kinase site and a focal adhesion targeting (FAT) site (59). There had been some controversy regarding the size of FAK. Guan and Shalloway (30) later found that the 120 kDa protein was identical to the 125 kDa protein. By using NIH3T3 cells and putting them on various plates with or without fibronectin (a ligand for beta1 integrins) they showed that the 120 kDa protein and the 125 kDa move at the same rate, translocated similarly and also bound similarly to c-Src. They suggested that the differences may be due to different regulators, i.e. 125 kDa is regulated by IN and 120 kDa is regulated by intracellular mechanisms. But another important outcome from this study is that it showed that IN activation, by the changes that occurred with the cells along with the presence of fibronectin, caused phosphorylation of FAK as seen by Western blotting. FAK has also been shown to be directly linked to beta1 and beta3 subunits of IN in an *in vitro* assay (90). By using chicken embryonic cells (CE) and genetically cloning the binding templates of multiple beta integrin subunits, Schaller and colleagues were able to show that FAK specifically

targeted the beta1 cloned peptide template. This is especially important considering the beta1 are in SM.

In a paper by Tzima (105), it was demonstrated that IN could be activated by SS, increasing both ligand affinity and binding to the extracellular matrix (ECM) in alpha5beta3 IN. Tzima perfused bovine aortic endothelial cells (BAEC) with a buffer and kept the cells either static or exposed them to SS that is consistent with physiological levels for arteries. This type of IN activation may be especially important when considering EE and the stress put on the muscles by the hypermaximal load. IN clustering has also been shown to activate tyrosine kinase proteins, which can activate FAK (50). In this study, the authors used a human epidermal carcinoma line of cells (KB cells) that had been incubated in a solution containing specific antibodies that, when looked at by fluorescent microscopy, induced IN clustering. After running these samples with SDS-PAGE, it showed that those cells which were clustered had a higher than basal level of FAK activation.

FAK Phosphorylation

It has been shown that FAK's main activation site is carried out at tyrosine (tyr) 397 and that this site is activated by autophosphorylation (89). Schaller was able to find this out by creating an amino acid genetic map and creating mutants at the various spots they believed the autophosphorylation took place. This autophosphorylation seems to be integral in the binding of c-Src to form the FAK/Src complex (19). By using CE, Cobb was able to genetically alter and control transcription and expression by transfection with a virus to encode FAK or c-Src. After lysing, they were able to do protein analysis by

SDS-PAGE. The results showed that FAK and c-Src, specifically the SH-2 homology, were binded well. X-ray chrystallography showed that two of the four main sections of FAK, the N-terminus FERM sections and the kinase domain, are the ones responsible for autoinhibition (59). In the autoinhibition mode, the various lobes of the FERM subunit bind with the kinase domain, effectively blocking tyr 397, which lies in between the two. The authors explain that the mechanism for autophosphorylation is still unknown although they state that it appears that a slight shift in FERM, possibly caused by IN signaling, is all that is required for the activation of the tyr 397.

There is also another major site that are responsible for full kinase activity of FAK and they are often placed together and mentioned as tyr 576/577. Calalb, et al., showed that this site is activated by c-Src. Using BALB/3T3 fibroblasts and creating a tryptic phosphopeptide map with labeled FAK, they were able to determine the activated sites by showing broken down FAK complexes. They then used DNA plasmids and infected COS-7 cells. By running the potential sites with an array of electric tests and affinity and structural tests, they concluded that this previously undetermined site was tyr 576/577 (14). By using FAK-null cells that were transfected to create various types of FAK Owen and colleagues (79) were able to show that tyr 576/577 were need for full activation of FAK. When there was a mutation on the tyr 397, there was no activation and cell motility and spreading decreased. With a functional tyr 397 and mutated tyr 576/577, motility and spreading were also decreased, although not to the same degree. This shows that activation of tyr 576/577 is necessary for full function of FAK. It appears this activation has to happen after tyr 397 activation. The FERM region effectively blocks the activation loop that contains tyr 576/577. Once tyr 397 becomes

activated it destabilizes the kinase domain enough that the activation loop can be phosphorylated (59). The role of FERM can be seen with another experiment. In a study using HEK 293 cells and FAK null cells that were infected to generate a tagged FAK, they truncated the FERM region of the protein and saw that there was in increase in overall activation of tyr 397. Even more, the mutated FAK was still able to transduce IN related signals via a replication in which the cells were suspended over 30 minutes and then placed on a plate of fibronectin (42).

FAK and Shear Stress

Helmlinger, et al., have shown that both pulsatile and constant SS can increase calcium levels in a cell (35). In this study, the authors placed bovine aortic endothelial cells (BA) in a solution along with a small capillary tube that made them able to control flow strength and frequency. The results indicated that constant and oscillating forces can increase calcium concentrations in the cell. To further this hypothesis, it appears different cells have an intrinsic optimal frequency in which outside pressures do not matter as long as this pulsatile frequency is met (4). By using a simple set up that the authors called a perfusion bioreactor, they were able to set up a solution resorvoir that was then pumped out and through a set of eight channels, flushing solution into contact with the cells, and then returned back to the resorvoir. By doing this, they were able to control how fast the pump sent fluid out and at what frequency, ultimately controlling the shear force. These cells multiplied efficiently at a rate very similar to rat lung epithelial cells and both of these differed quite a bit from rat small intestine epithelial cells. This activation may have significant outcome. It shows that there are intraspecies difference, meaning that different cells in the body react differently to SS. For example, in

endothelial vascular cells there is a constant physiological SS upon them at all times. This SS has been shown to activate FAK in these cells (58). Li and colleagues also used aortic endothelial cells with a steady flow across the cells. There was a marked increase in phosphorylated FAK after one minute that gradually faded over the course of an hour. This has also been demonstrated with colon cancer cells. Thamilselvan, et al., (96) have shown that FAK is autophosphorylated in colon cancer cells that were exposed to increased pressures. By controlling ambient air pressure in an airtight container, they showed that increasing the pressure induced FAK activation. They also showed that increasing the calcium concentration in the cells increased FAK activation. For further diversification of FAK, it has been shown that SS resulting in osteoblast MT causes the de-activation of FAK related proteins (112). Osteoblast-like cells were hit with fluid pressure to mimic MT. With varying percentages of functional FAK, certain downstream proteins were severely ablated, showing that FAK is necessary in communicating during MT. Again, this is very important when considering the type of stress that SM deals with during eccentric loading.

FAK and Cardiac Hypertrophy

The beta1 IN subunit plays a significant role in cardiac hyptertrophy. By using neonatal rat ventricular myocyte (NRVM) cell cultures with increased cell volume caused by phenylephrine, Ross, et al., (85) found a genetic increase in beta1 subunits caused an increase in cell hypertrophy when using atrial natriuretic factor (ANF), a sign of hypertrophy, as a marker. This shows that the increase in beta1 subunits can control intracellular hypertrophy signals. To further elucidate the role of FAK in hypertrophy Torsoni, et al., (101) looked at possible genetic effects that FAK would have in a

hypertrophic model. They used NRVM cell cultures and exposed them to a device that stretched them to 115% of their basal length at a frequency of 1 Hz. They found a gradual increase in activated FAK activity over time. By mutating tyr397, stretch-induced activity was attenuated. The study also showed that the mutated FAK had a marked decrease in ANF-promoter activity, meaning the FAK could be responsible for hypertrophic gene expression via its signaling cascade. Continuing with hypertrophic models, a study by Franchini, et al., (25) found that FAK becomes elevated after increasing aortic pressure in rat hearts. Using Wistar rats, they increase blood pressure by putting on an aortic clamp for 60 minutes at 60 mmHg or in a graded fashion that increased blood pressure every 10 minutes. In the graded test they saw a rapid increase if FAK phosphorylation. For the constant clamp, FAK remained constant. A different study by Torsoni, et al., (102) found that stretching myocytes increases FAK activity similarly to the previous study. Using NRVM, they found that by stretching the cells at the same protocol used above gave similar results. However, they also showed that FAK may have a precursor that is different than simply IN signaling. By inhibiting the RhoA/ROCK pathway, stretched-induced FAK activation fell significantly. A correctly functioning RhoA/ROCK pathway maintains a stable actin organization so this means that if there is an no stable actin on the cell membrane, FAK activity will falter. Finally, in a study by Marin, et al., (64) it was shown that inhibiting Shp2 in NRVM, which the authors claim can dephosphorylate activated FAK and lead to apoptosis, increases FAK activity. In stretched cells, Shp2 was significantly decreased as FAK activation increased, implying that Shp2 can moderate FAK activity. They also showed that inputing a silenced Shp2 RNA in non-stretched cells leads to higher levels of FAK

activation. Furthermore, when FAK/c-Src complex was formed, there were higher levels of the hypertrophic proteins Akt and p70s6k.

c-Src

c-Src (pronounced "Sarc") is a 60 kDa protein and, similarly to FAK, is a non-receptor tyrosine kinase that is located within the cytosol (84). This protein should not be confused with v-Src, a viral protein originally found in chicken tumors in 1911 (84). c-Src is a member of a larger family of highly conserved protein kinases called Src-family kinases (SFK), of which there are 11 in humans (62). c-Src can be broken down into six domains which all have different actions when activated in the cell: Src homology (SH) 4 which starts at the N-terminal, SH3, unique domain, SH2, the kinase domain (also known as SH1), and the C-Terminus. All members of the SFK have SH4, SH3, SH2 and SH1 in common but their exact amino acid structure differs slightly allowing for the different characteristics of the family (81). c-Src SH3 and SH2 are necessary for binding with FAK and downstream substrates activation is dependent upon this complex forming (97). This happens once the autophosphorylation of the active binding site of FAK, tyr 397, becomes open, which then opens tyr 576 and 577, both of which are mediated by Src (14). Two tyrosines at 576 and 577 were replaced with phenylalanines and the level of FAK activation was measured. This switch in amino acids caused a significant decrease (about 70% compared to wild type) in FAK activity. The following are brief descriptions of the individual aspects of the main domains of c-Src.

SH4

SH4 is a naturally unfolded domain (also known as intrinsically unstructured or unfolded proteins) and this is thought to make it easier to transduce signals by increased flexibility and binding ability (27). SH4 is mainly responsible for most SFK's membrane attachment by transforming the chemical binding sites on the fatty acid part of the cell membrane (103). The actual mechanism behind this is the covalent binding of a myristate (a 14-carbon fatty acid) to the N-terminal and the binding/esterification of a palmitate (a 16-carbon fatty acid) to the cysteine residues on SH4 [for in depth review see (93)]. However, c-Src is a non-palmitating SFK that rapidly moves from the plasma membrane to the intracellular organelles, which differs from many of the other SFK's and may explain differences in SFK signaling (47). SH4 is not only responsible for anchoring the cell. It may also be responsible for plasma membrane blebbing (cellular swelling caused by disorganization of actin) by controlling downstream proteins in a c-Src-dependent manner (32). The importance of this is that blebbing of the cell may be a sign of apoptotic or necrotic events (6). Blebbing has also been shown recently to be a sign of increased cytokine release, mainly interleukin (IL) 6 and tumor necrotic factor-alpha (TNFa), when compared to apoptotic cells that were not blebbing, and to activate T-cells (26). c-Src has been shown to be necessary for increased cell motility, a possible mechanism for mestasis of cancer cells (104).

SH3

SH3 is a small domain of 60-80 residues that is made up of three loops in SFK and binds to proline rich areas of other proteins (16). SH3 can have a prominent role in the progression of cancer. Binding of endothelial-derived gene 1 (EG-1) has been evidenced to happen fairly easily on the SH3 domain of Src (61). In this study, Lu and colleagues

used human embryonic kidney (HEK) 293 cells to see the interaction of SH3 and potential binding abilities with c-Src. They found that 21 proteins had a 2.5 fold or more increase in SH3 binding with EG-1, with c-Src having a 4.8 fold increase. Furthermore, they found that elevated levels of EG-1 correspond with activation of c-Src by decreased levels of tyr 419, the active site in human cells. Elevated levels of EG-1 have been witnessed in breast, prostate and colon cancer cells when compared to benign cells (113). SH3 binding to ADAM12-L, a metalloprotease shown to have a role in tumor progression, has also been attributed to activating c-Src. This may start signaling cascades that deal with mechanically altering the cytoskeleton, proliferation, protein migration or keeping the activation of c-Src for a longer duration (94). If this binding does occur and causes prolonged activation of c-Src, then it's possible that c-Src may have a larger impact on cell status than normal and possibly inducing cytoprotection. SH3 may also be responsible in binding c-Src to calcium channels, as a deletion of SH3 decreased c-Src binding to calcium channels in human smooth muscle, which decreases calcium channel activity, possibly causing pathologies such as inflammation of the colon, which has attenuated calcium channel function (45). However, the authors stated that SH2 is probably able to alter calcium channel function without SH3 binding. Higher cellular levels of calcium has been shown to increase c-Src and is also regulated by SH3 (68). This may have an effect of regulating other pathways, especially if considering SM. SH3 binding may also play a role in decreasing contraction velocities in a dog myocardial infarction model (49). But perhaps the most important role SH3 does is help in covering the main activation site of c-Src and, along with SH2, control the activity of the protein in a manner accepted as the switch, the clamp and the latch (33). c-Src has four main

sections involved with this inhibition: SH2, SH3, two kinase lobes that make up the kinase domain, a N-terminus lobe and a C-terminus lobe, to which the terminal tail containing tyr 527 is attached (84). The latch part indicates that the inhibitory and phosphorylated tyr527 on the C-terminus of c-Src is directly binded with SH2, fully attaching SH2 with the C-kinase lobe and forcing SH3 into contact with the N-kinase lobe. All of this together creates the clamp over the activation site. The switch is merely the dephosphorylation of tyr527, opening the activation site to the cytosol (See Figure 2.1).

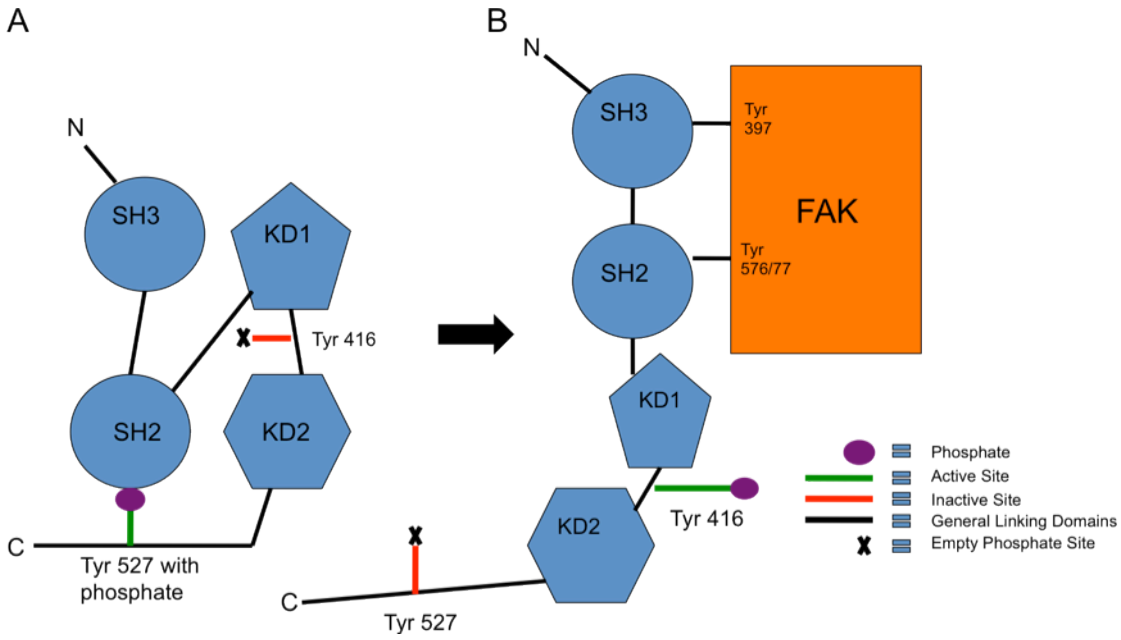


Figure 2.1: Activation of c-Src. *A)* A phosphate group on tyr 527 "latches" to Src Homology (SH) 2, causing the "clamp" and keeping the active site buried within the protein and rendering c-Src inhibited. *B)* Activation of c-Src is caused by a "switch" in phophorylation of tyr 527. Once the phosphate group is released, the protein linearizes and opens tyr 416 to the cystosol where it is quickly phosphorylated and becomes active. Figure depicts active c-Src binding to an active FAK on tyr 397 and tyr 576/77. Adapted from (84).

SH2

SH2 is a domain that contains approximately 100 amino acids. As previously stated in the above section, SH2 is quite active in the self-inhibition of the whole protein. However, SH2 has many roles outside of inhibiting the phosphorylation of c-Src. In another very important aspect, SH2 is shown to be the main player in binding to FAK. Cobb, et al., (19) showed that in chicken embryonic cells (CE) that had specific genetic deletions, SH2 was the main binding domain. By replacing normal SH3 sequences with differing variations but each having its own respective normal SH2 sequences, there was still stable binding to FAK. But when they reversed the homolog variants so that SH2 was altered and SH3 was normal, there was no binding to FAK. Another role of SH2 is the phosphorylation of the p110 subunit of PI3K requires the SH2 domain of c-Src (43). Although the authors are fairly confident that it is c-Src, they do give a little leeway by stating that it could be any of the SFK. In this study a prostate cancer cell line that was PTEN deficient (PTEN is a regulator of PI3K). After confirming that other proteins had not phosphorylated PI3K in the cells, a c-Src inhibitor was inserted into the cells and the authors saw the dissolution of the subunit p85 from p110 while also seeing a marked decline in the p110-beta. Interstestingly, there was not a substantial decrease in Akt phosphorylation, meaning that the activation of p110 by SH2 is not required for signal propagation downstream of PI3K. The last act of SH2 that will be covered is its role in cell motility, and its ability localize to focal adhesion on the plasma membrane and activate FAK (111). In this multiple experiment study they demonstrated that by genetically altering an arganine on position 175 there was a decrease in localization of

shortened SH2s to focal adhesions, decreasing binding of the homolog to FAK. They compared this with an abbreviated c-Src that had a fully functional SH2 region but no kinase domain and there was full binding to FAK. They then used a full c-Src but with the defect still in the SH2 and the showed that there was little binding and little cell motility, implying that SH2 is necessary not only for binding, but for guiding the protein to the focal adhesion.

SH1 (The Kinase Domain),

The kinase domain (SH1) contains the main activation site for c-Src, tyr 416. c-Src 416 is the chicken numbering and it is the literature standard because it was found in chickens first (so is tyr 527, the main inhibitory site) even when using human cells (84). For example, when using even human samples, which have the main activations and inhibitory sites on tyr 419 and tyr 530, respectively, they are referred to as tyr 416 and tyr 527. As previously mentioned, when tyr 527 becomes dephosphorylated , it allows tyr 416 to open to the cytosol and become ready for activation.

It has been shown that c-Src can be autophosphorylated. The first study to show this was done by purifying c-Src by immunoaffinity from European field vole 100,000 fold. Labeled ATP was then added to the precipitate and the phosphorylation rate was measured via SDS-PAGE (83). In 1995, Osusky showed by an autokinase assay that site 416 of a wild type c-Src has an autophosphorylation affinity approximately 10x (in a solution with 26 mM Mg/ATP) than that of site 527 on a mutated c-Src with an amino acid change on tyr 416, demonstating the protein prefers to be activated (78). But it also shows that by being able to control its own inhibition site, c-Src can actually down

regulate itself if the concentration of ATP is high enough. When the concentration of Mg/ATP was increased, phosphorylation of tyr 527 increased, inactivating the protein. Another interesting finding from this study is directed to whether autophosphorylation happens via intra or inter molecular activity. By purifying extracts to the point where they could not identify other tyrosine kinases they placed in the extract in differing dilutions of a phosphate buffer. They assumed that if it was intramolecular there would be constant rates despite increasing concentrations and and if it were intermolecular it would be linearly related to the increasing concentration. What they found was tyr 416 was shown to be mostly intermolecularly phosphorlyated while tyr 527 in mainly intramolecularly activated.

The activation of tyr 416 can be seen in a study using hepatocyte growth factor (HGF) in HuCCA-1. When exposing these cells to HGF, there was an increase in cell proliferation and tumor invasion. This corresponded to an increase in c-Src/FAK complexes. Interestingly, the experiment showed that the c-Src/FAK complex was formed before FAK phosphorylation by looking at the time course of activation (82). However, inhibition of c-Src has been shown to decrease mestasis in breast cancer cells. By creating activated or unactivated cancer genes and infecting them into mice to look at the effects *in vivo*, Rucci and partners used a c-Src pharmacological inhibitor to see the effects on lethality and tumor size. They saw a 2.5 decrease in mortality in the animals that had the inhibitor injected and there was no behavior that would indicate that the injection was harmful. In a mouse strain in which there was a kinase dead dominant gene with a mutation to tyr 527 (to ensure very small rates of phosphorylation) there was a

decrease in tumor size and mestasis, indicating less cancer activity. The authors claim that this may yield a potential therapy to breast cancer patients (86).

As with FAK, activated c-Src can play a role in cardiac hypertrophy. In a study that used a treatment of Endothelin-1 (E1) it was shown that EI did not increase total c-Src but it did increase phosphorylation of the active site by immunoblotting. To confirm this they used a c-Src autophosphorylation assay and a general c-Src activation assay and found that treatment of E1 had a seven-fold increase in c-Src activity. Using the ANP marker for cardiac hypertrophy, the study showed an increase in ANP promoter activity and this was decreased 50-60% with c-Src inhibitor Csk (51). Finally, using cat cardiac myocytes that had been exposed to an increase in pressure-load, Kuppuswamy, et al., saw in increase of activated c-Src at the cytoskeleton, indicating that cellular membrane and IN dynamics may play a role recruiting c-Src for downstream signaling (54).

The C-terminal Tail and the Role of Csk

The C-terminal tail (CTT) contains the inhibitory site, tyr 527, which is phosphorylated by C-terminal Src kinase (Csk). As stated before, in the inhibitory state of c-Src, tyr 527 binds to the SH2 region, effectively locking tyr 416 in the middle of the protein. Okada and Nakagawa (77) were the first to see that a protein kinase phosphorylated tyr 527 in a non-truncated protein. By purifiying non-neuronal and neuronal c-Src from neonatal rat brains and breaking them down, they were able to see a protein that co-migrated, along with a peptide created to bind to CTT. This protein that co-migrated they named Csk. In 2002, Ogawa and colleagues were able to determine the structure of Csk via crystallography. What it showed was a structure very similar to c-Src. There was an

SH2, SH3, and a very similar kinase domain that also had two lobes. The main difference was the SH2 was located in the top left of the N-terminal kinase domain, where c-Src SH2 is located near the C-terminal kinase domain (76). Later, the Nakagawa lab discovered that an unidentified protein negatively regulated c-Src at the tyr527 site (71). They used neonatal rat brain c-Src again and mutated the try527 site to a phenylaline. They saw that there was an increase in c-Src activation in the presence of Csk. To confirm this, they coded the gene and found that the sequence matched well. They also saw that there was no autophosphorylation site like most SFK's and there was a stop codon where tyr527 should be. They mentioned that this could be very important to its regulatory and substrate reactions. Nada, et al., showed in vivo that Csk was a negative regulator of c-Src. Using Csk-deficient mouse embyros, they were able to show those embryos that lacked the ability to code Csk had increases in c-Src activation (72). By the use of flouresence resonance energy transfer (FRET) it was found that Csk binding protein (Cbp) is responsible for an activated Csk being recruited to the cell membrane where c-Src is located (66). Levinson, et al., showed by crystallography of activated Csk and c-Src that the active region of Csk binds very close the the C-terminal tail and the try527 of c-Src is in a desirable position for easy access to the donor proton (57). These authors also showed, by surface plasmon resonance, that Csk can differentiate between active and inactive c-Src. They placed an actived sequence of the CTT c-Src into the kinase domain and saw that there was no increased binding of Csk. They concluded that this was so there was no competition between the active and inactive tail fighting over Csk.

Now that c-Src and FAK have been discussed about in myriad cell types, the focus of the paper will now switch to proteins that have been studied extensively in SM: PI3K and the main proteins that are downstream of it, AKT and mTOR. There will also be a focus of ERK 1/2 and certain Hsp's.

PI3K/Akt/mTor Pathway

PI3K

PI3K is made up of two subunits: a p110 catalyzing subunit and a p85 regulatory subunit. Interestingly, in 1991 Escobedo, et al., discovered that p85 has an SH2 subunit. Using BALB/c 3T3 cell lysates, they were able to purify p85. By exposing their extract to SDS-PAGE and cutting out the correct bands they were able to clone the DNA. What they found was that the p85 subunit had an SH3 area and two SH2 subunits (24). Then in 1992, a mechanism for PI3K p85 activation was discovered. Hadari, et al., used live liver cells that had been injected with hydrogen peroxide and vanadate (inhibitors of certain tyrosine kinases that give rise to similar effects of insulin) or insulin. After 15 minutes the rats had their livers removed. What they saw was the p85 subunit bound to a phosphorylated region of IRS-1 in an immunoprecipatate, meaning that p85 of PI3K is attracted to proteins with a phosphorylated tyr (31). Why this is important can be seen with a study completed by Chen and colleagues. They were able to use mutated and WT tyr 397 sites on FAK to determine that the SH2 region of p85 of PI3K can bind to the main activation loop of FAK and result in activated PI3K. The authors mutated the various tyr phosphorylation site with phenalynines. They noticed that p85 binding disappeared when the mutation was on the tyr 397 site (18). This is important

considering that autophosphorylation of FAK now can direct traffic in a completely different manner because PI3K is attracted to these activated tyr. In a study using fibronectin (an IN activator) covered plates and using human thyroid cells, it was possible to see that there was an increase in PI3K cells. When wortmannin, a potent P13K inhibitor, was added the cells became apoptotic, demonstrating the IN-based PI3K activation is crucial for cell survival (40). To see how mechanotransduction Sasai, et al., (88) demonstrated how mechanotranduction alters PI3K. 13-day old chicken breast muscles were stretched at 1/6 Hz to 110% of their length. These authors saw an increase in cell size that they attributed to the PI3K pathway. When they added wortmannin they saw a 30% decrease in size in control muscles and a 25% decrease in size from the stretched cells, showing that PI3K is necessary for stretch-induced muscle growth. In another example, Zhou, et al., used aortic stents to demostrate that stretch induces PI3K. By placing stents they were able to look at how stretch can affect vascular smooth muscle. They administered wortmannin for two days before the stent were put it and they kept administering until the rats were killed. In the wortmannin-injected rats, there was a significant decrease stretch-induced downstream proteins, demonstrating again that PI3K is necessary for stretched-induced hypertrophy signaling (114).

Akt

In studies in 1996 and 1997, Alessi and colleagues went about working on the sites and mechanisms of Akt activation. In the former study, they used L6 myotubes to determine Akt activation sites in the presence of insulin and/or wortmannin. They saw that with insulin, IGF-1 and wortmannin added to wild type or cells with kinase dead or altered threonin (thr) 308 and altered serine (ser) 473 sites that significant activation only

happened with IGF-1 and insulin in the wild type, showing that you need both sites for complete activation and that activation is PI3K dependent. By mapping these sites after a tryptic digestion, they were able to confirm that they were the major activation sites (1). Allesi, et al., (2) found that since wortmannin inhibited the signal there might be something responsible for activating Akt downstream of PI3K, specifically its product PIP3. They discovered this from using purified rabbit skeletal muscle and single line coding for the region of thr 308 and assaying the reactivity with PDK1, the protein they discovered activated thr 308 and that activation was enough to activate ser 473. Toker and Newton (100) then found the same by using two versions of pure Akt, one active and the other rendered inactive by temperature, and a pure PDK1. Since it is known that thr 308 is activated by PDK1 it is possible to see if ser 473 is autophoshorylated. The results showed the ser 473 activation mirrored that of thr 308 confirming that ser 473 is autophosphorylated. Then in 2005 it was shown that the elusive "PDK2" is actually a complex that contains mTOR and rictor (mTORC2). Using HEK 293 and HeLa cells with variations of rictor and mTOR, they showed that ser 473 activation was dependent on both of them. It also showed that the presence of PDK1 with mTORC2 had greater activity than just PDK1 alone, implying that ser 473 may be activated first (87).

Sasai (88) and Zhou (114) saw increases in Akt activity. Gayer, et al. used a Flexcell machine to deform human intestinal epithelial cells in a cell culture. They were flexed at a 1/6 Hz frequency at 110% resting length. Their findings showed that p85 substrate is activated and Akt becomes more active over time in strained cells. They also showed a role of c-Src acting as an upstream mediator in strained cells. With the use of PP2, a c-Src inhibitor, they saw a return to normal levels of pAkt and activated p85 in strained

cells, hinting that Akt and PI3K activation is downstream of a c-Src dependent mechinism in strained cells (28). Upregulation of Akt via beta1 integrin subunits has been shown to protect cells from apoptosis. Tian, et al., used collagen gel contractions as a means of showing apoptosis in human lung fibroblasts. The cells with higher levels of collagen showed better structure than those with minimal amounts. In those cells with low levels up collagen, Akt was upregulated by a virus and these cells were tested for levels of anoikis, a type of apoptosis, and it was shown that the upregulation of Akt protected the cell from self-termination (99). Furthermore, Katta, et al., demonstrated the mechanical stress from EE in Zucker rats increase activation of Akt in lean rats. By attaching an electrode to the sciatic nerve they were able to simulate eccentric loads in the rats. Their results showed that both active site of Akt were activated directly after exercise and then three hours later and that muscle contractions can induce Akt activity (48).

mTOR

mTOR is a large protein that can interact with various other proteins. There are two forms: mTORC1 and mTORC2. mTORC1 has a rictor module which allows it to activate Akt and MTORC2 has a raptor molecule which is activated by Akt. In a study done by Navé, et al, HEK 293 cells were used to monitor the appearance of mTOR in the presence of Akt, insulin and wortmannin. They saw that labeled mTOR increased phosphorylation on its ser 2448 site when combined with Akt. They also saw a decrease in concentration of activation mTOR in the presence of wortmannin, indicating it is somewhat PI3K dependent and furthermore that ser 2448 activity did not go down in the presence of rapamycin, showing that ser 2448 is not that major attack site of rapamycin

(73). In 2007, it was hypothesized that there could be another direct inhibitor of mTORC1. Wang, et al., used HEK 293 cells and immunoprecipitated them and then exposed them to protocols that could control the amount of expression they had. The results showed that in mTORC1 there was a distinct band at 40 kD and mass spectrometry revealed that it was proline-rich Akt substrate (PRAS40) (106). They also showed that the presence of insulin could disrupt the binding of PRAS40, demonstrating it as a negative regulator of mTORC1. Kovacina, et al., were able to show a few years earlier that PRAS40 was actually an Akt substrate by treating it with insulin, rapamycin and wortmannin. There was no effect from rapamycin compared with insulin, showing that the target is upstream of mTOR and its activity was shut down with wortmannin, showing its downstream of PI3K (52).

In a recent study, O'Neil and colleagues used an *ex vivo* EE protocol with rats in which the EDL muscle of the rat was mechanically loaded and an *in vivo* protocol where they attached an electrode to the sciatic nerve and contracted electronically. Using mTORC1 downstream target p70S6K (p70) as a marker of mTORC1 activity, they observed that EE raised activation of p70 even in the presence of wortmannin, indicated that this increase is PI3K independent (75). These results followed similar results from a previous study. In 2003, Bolster, et al., used rats trained to reach up and grap a high bar, simulating concentric and eccentric contractions. They saw an increase in mTORC1 ser 2448 activation and also p70 after 10 minutes, showing that muscle contractions can induce these proteins (9). SS can also cause mTOR activation. Using an oscillatory mechanism and HUVEC cultured cells, the authors saw in increase in p70 activity. They

also showed that the addition of rapamycin stopped flow-mediated DNA synthesis, showing a role of mTOR in a flowing medium (53).

ERK1/2

ERK1/2 and MEK

ERK1/2 is a 42 and 44 kDa protein that is abundant in all cells throughout the body. Its main activation sites were discovered by Payne, et al.,. By using a labeled Swiss 3T3 cell that had been activated by insulin to assure phosphorylation, the researchers were able to digest the protein with trypsin, keeping intact the activation sites, and then mapped and sequenced it. They found that phosphorylation occurs at thr 183 and tyr 185 on ERK2 (80). But a question lingered after the Payne study. They believed that something, either autoregulation or a protein kinase, was acting on ERK2. A year later, Crews and Erikson (20) found the answer. They were able to purify a protein kinase that they termed MAPK/ERK kinase (MEK) in murine cells. After purifiying this protein, they saw a 33 fold increase in ERK2 activity using a labeled protein and SDS-PAGE. In a study by Butch and colleagues, they looked at the activation of ERK1. They mentioned that the activation site for ERK1 is a thr 202 and tyr 204 and the sequence around these is the same as in ERK2, suggesting that MEK should show no difference towards either site. By mutating the area around the activation site they noticed that basal levels of activation decrease drastically with mutations to try 204 and thr 202. A very interesting result was a mutation on the tyr 208 increased phosphorylation by 400 percent but has zero effect on activity, an according to the authors this means that the site is necessary for activation of thr 202 (13).

ERK1/2 Activation

Dvir, et al., looked at how fluid flow can affect ERK1/2 activity. By placing rat neonatal myocytes in a bioreactor, they were able to push fluid over the cells at 1, 2 or 3 Hz frequencies. They used the presence of angeotensin II as a positive control because it activates ERK1/2. ERK1/2 activation increased dramatically in the pulsed cells compared to a static control. However, differing flows had no effect on the activation (22). Schwachtegen, et al., (91) used HAEC and exposed them to varying flow rates. The authors examined *egf-1*, a gene downstream of ERK 1/2. With increasing flow pressure and time exposed to the flow, they saw an increase in the *egf-1* gene promoter, demonstrating ERK1/2 activation. They then exposed the cells to a MEK inhibitor and all results were ablated, indicating that ERK1/2 is necessary for this genes expression in dynamic cells. Furthermore, in human lung epithelial cells it has been demonstrated that FAK and c-Src may be mediators in ERK1/2 activation. By placing these cells on elastomere membranes and exposing them to a computer driven vacuum, they were able to stretch the cells. What they saw was an increase in proliferation in strained cells compared to static cells at each time point. They also saw an increase in ERK1/2 activation. However, when the cells were exposed to PP2, a c-Src inhibitor, they saw a decrease in ERK1/2 activity while no decrease in FAK activity, showing that ERK1/2 is a downstream player of c-Src in strained cells (17). The final study that will be covered deals with EE and how it plays a role with ERK1/2 activity. In a study that used rats with electrodes attached to their sciatic nerve, researchers looked at four different contraction protocols and ERK1/2 activation. By contracting the muscles to different displacments at

a constant frequency, the researchers were able to show that EE generated the most ERK1/2 phosphorylation compared to the other protocols (65).

Hsp 25/27 & 70/72

Hsp's are a highly conserved group of proteins that respond to cell stresses such as (obviously) increases in cell temperature, incorrectly folded proteins and exercise. While there are many mechanisms and members of the Hsp family, this section will focus on Hsp 70/72 (human/rat) and 25/27 (rat/human) and EE. It is generally accepted that Hsp 70 and Hsp 25 can disrupt aggregation of proteins and chaperone them away while also folding malformed proteins [for reviews see (36) and (110)]. Hsp's could help in stabilizing the cell during EE by refolding membrane proteins (integrins) and/or chaperoning them towards the stress.

Hsp 25/27 and FAK

While no published studies were found dealing with IN function and Hsp directly in SM, it is possible to see an interaction with Hsp and IN signaling via cell culture and cardiac tissue. In NIH3T3 cells overexpressing Hsp27, there was a greater presence of phosphorylated FAK and c-Src compared with control cells and an increase in focal adhesions. There was also increased adhesiveness of the Hsp treated cells. Furthermore, when a protein that disrupts actin organization was added, Hsp27 activation of FAK disappeared (56). This insinuates that binding to the cytoskeleton is crucial and further implicating IN signaling. In ischemic rat neonatal ventricular myocytes it was found that heat shocked cells increased IN/FAK binding (108). By immunoprecipitating the β1 subunit and probing it for FAK activity there was a significant increase in IN/FAK

binding at 10 min into ischemia. It has also been found that heat shocked rats have increased FAK activity in left ventricular tissue meaning that FAK may serve as a protective mechanism against cell stress (107).

Hsps, FAK and Anti-apoptosis

Hsp 70/72 is an anti-apoptotic factor that gets upregulated during cellular stress. One of the ways in which it protects against apoptosis is its ability to protect FAK. In possum renal cells, Hsp 72 was overexpressed by heat treatment or viral transfection and incubated with caspase-3, an apoptotic protein that fragments FAK. In cells that had been overexpressing Hsp72 and incubated with caspase-3, those incubated with FAK before caspase-3 was added were preserved more and fragmented less than cells that had been incubated before FAK was added (63). It was also shown that Hsp 72 coimmunoprecipated with FAK, providing evidence that Hsp 72 may preserve FAK by physically binding to it and preventing its degradation. Hsp 27 can also be upregulated in an anti-apoptotic manner. By exposing renal endothelilial cells to a cytotoxin, Hsp 27 was found to upregulated the fastest of the Hsp family and it was located at sites of high focal adhesion turnover (21). In these cells they also inhibited p38 activation of Hsp 27 and saw a significant increase in cell death and loss of cellular adhesion. Cells that were overexpression Hsp 27 had increased cellular adhesion and cell death was partially ablated when exposed to the cytotoxin.

Hsp and EE

Huey used a functional overload model using rats to determine if a constant overload (similar to EE) would increase Hsp 25. By removing the gastrocnemius, she was able to

make sure there was a force overload on the plantaris and soleus muscles. The contralateral leg was unaltered to be used as a control. After 3 or 7 days the muscles were removed. The results showed that were was an upregulation of Hsp 25 expression and activity in both muscles compared to the control. There was also increased activity in the 7 day versus day 3 (38). In 2000, Thompson and workers looked at Hsp 27 and 72 in biceps brachii following a single bout of EE. Using a Biodex arm curl machine, the non-weight trained subject had to do 2 sets of 25 reps with their non-dominant arm and biopsies were taken 48 hrs post. The study showed a 10 fold increase in the Hsc/Hsp 70 complex (they mentioned that the antibody used had a larger affinity for Hsp 70 not Hsc) and a 2.4 fold increase in Hsp 27 (98). We can also look at this from a non-supramaximal test. Morton, et al., performed a 45 min, non-damaging treadmill test at a percentage of the lactate threshold of active but non-trained subjects. They had vastus lateralis biopsies at 1, 2, 3 and 7 days post. Hsp 72 peaked at day 2 and was significantly higher day 7. There was no change in Hsp 27, suggesting that Hsp 27 may be important in helping after muscle damage has occurred and that at submaximal levels, Hsp 70 can handle the load (69).

Conclusion

Cells face amazing amounts of stress on a daily basis. The above are examples of proteins that may have some amount of activity due to SS or MT via IN signaling. While the first section of the paper dealt with almost every cell but SM, we can try to assume that if the protein works in a hypertrophic/prolific manner in a breast cancer cell, there is nothing to stop it in a SM cell after a bout of EE. So, hypothetically, we could say that during a bout of EE, MT causes the clustering and activation of INsThis activates and

localizes IN, which activates FAK. FAK could then activate the p85 subunit (with two SH2) of PI3K, which could cause its own anti-apoptotic and hypertrophic signals downstream via Akt and mTORC2. In another pathway FAK then recruits c-Src SH2 to its active site which in turn activates ERK1/2 in a hypertrophic, anti-apoptotic manner. FAK could also be protected by a bound Hsp 70/72 to prevent degradation from apoptotic proteins. Hsp27 also activates FAK and goes to focal adhesions to prevent cellular injury (See Figure 2.2).

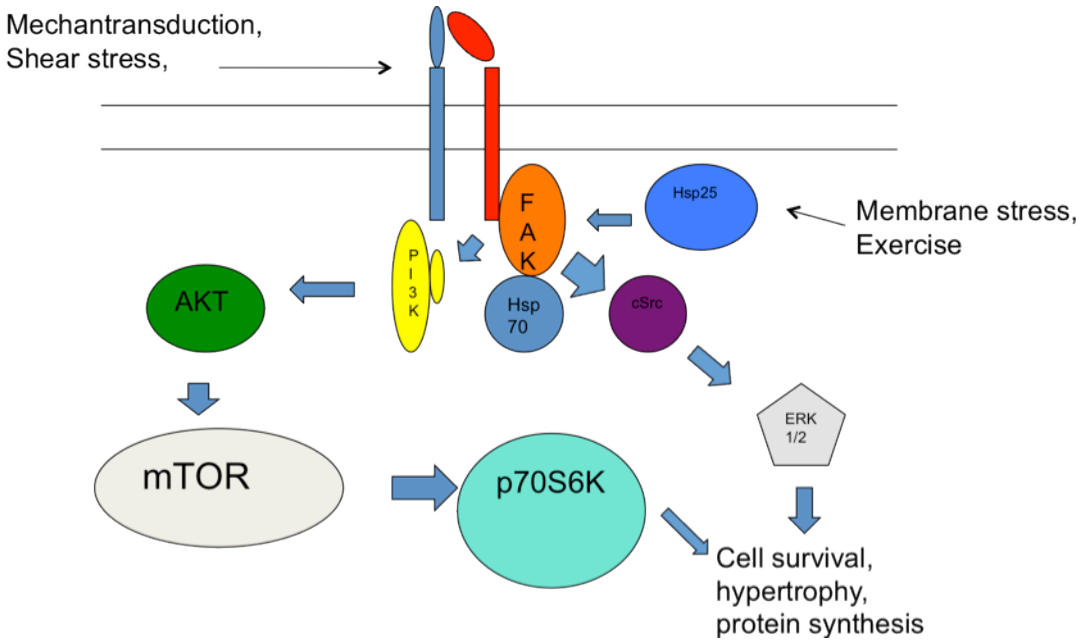


Figure 2.2: General Integrin Signaling Pathway and Heat Shock Proteins. Mechanotranduction and shear stress cause activation of integrins. This activation causes focal adhesion kinase (FAK) to become activated, causing the activation of two separate pathways, PI3K and c-Src, both responsible for cell survival. Heat shock proteins (Hsp) have been found in higher levels during membrane stress and exercise. Hsp 25/27 has been found to activate FAK and protect the cytoskeleton and Hsp 70/72 has been found to bind to FAK and prevent degradation.

Chapter 3

Methods

Introduction

The tissue used was taken from an archival source but a general overview will be provided. The study that originally used this tissue source was looking at the PI3K cell survival and hyptertrophy pathway and apoptotic MAPK pathway. The study also used hemotoxylin and eoson staining to determine macrophage invasion, nuclei location and muscle damage. Tissue was also used in caspase detection kits as a sign of apoptosis. Because of this only IN related signaling will be looked at in the current study.

Animals Used

Male Wistar rats were used. Each weighed between 300-350g before heat treatment. They were divided into a control group, an exercise only group (EE) (n=8-9) and heat shock group (HS)(n=8-9). The rats were fasted for 12 hrs pre-heat shock. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center (IACUC #1534)

Eccentric Exercise

Rats were randomly put into the EE or HS group. The exercise consisted of running at 18 m/min down a grade of -16%. The rats ran for 5 min with a 2 minute break immediately after. They performed this sequence a total of 18 times, totalling 126 minutes.

Heat Shock

Rats were randomized into a control group that internal temperature kepts at 36°C and a HS group in which the internal temperature was 41-41.5°C for 20 minutes. The rats in the HS group were anaesthetized and then set in a flotational suit. The rats were

immersed in 42° C water until their body temperature reached the desired internal temperature. Saline was injected afterwards to protect agains dehydration. The HS rats performed the exercise bout 48 hours after treatment.

Muscle Samples

The soleus, vastus lateralis white and vastus lateralis red were dissected at 2hr and 48hr post-exercise. They were immediately frozen in liquid nitrogen and then stored. The protein was extracted with a cocktail of protein extraction solution (T-Per Tissue Protein Extraction; *Thermo Scientific, Waltham, MA*) phosphotase inhibitor (Halt Phosphotase Inhibitor; *Thermo Scientific, Waltham, MA*), protease inhibitor (Halt Protease Inhibitor; *Thermo Scientific, Waltham, MA*) and PMSF (Nunc; *Nalgene International, Rochester, NY*). The muscle samples were placed in the solution and ground using a mortar and pestle and then centrifuged at 3000rpm for 3 min. Samples were measured for protein content with a BCA protein assay kit (Pierce BCA Protein Assay Kit; *Pierce, Rockford, IL*). Samples were then put into a ratio to equal 80μg/mL with a corresponding amount of HES buffer. A 5x lane marker was also added to each sample.

SDS-PAGE

Electrophoresis will be performed via SDS-PAGE (Mini-PROTEAN 3 cell and PowerPac High-Current Power Supply; *Bio-rad, Hercules, CA*). Proteins will be ran through a 5% stacking gel and varying separating gels (6.0% for beta1 subunit, c-Src and p-c-Src527 or 10% for ERK1/2 and pERK1/2) at .05 amps. After the protein marker has started to run off the gel the current will be stopped and the gel will be prepared for transfer.

Western Immunoblotting

Proteins will be transfererd onto a PVDF membrane (Amershand Hybond; *GE Healthcare, Buckinghamshire, England*) that has been activated in a 30s immersion in methanol. The proteins will be transferred at .20 amps for 110 minutes and be blocked immediately after in a 5% dry milk/TBST solution for an hour. After blocking, the membranes will be placed in a primary antibody (beta1 integrin; *Santa Cruz Biotechnology, Santa Cruz, CA*; c-Src, p-c-Src527, ERK1/2 and p-ERK1/2; *Cell Signaling, Beverly, MA*) diluted with a 1% milk/TBST solution. Primary antibody dulitions will equal 1:500-1:1000 (μL antibody:μL 1% milk solution). The membrane will be incubated overnight in a 4° C refridgerator. After incubation, membranes will be rinsed in 3x5 min washes in TBST and then placed with an anti-rabbit (all proteins are anti-rabbit) horsheradish peroxidase linked (HRP) secondary antibody (Anti-rabbit IgG; *Cell Signaling, Beverly, MA*) with a 1:2000 dilution for an hour. Following incubation membranes will be washed with a 2x5 min set with TBST and then 1x5 min with TBS. Membranes will be incubated with a HRP chemilumenescent (Amersham ECL Western Blotting System; *GE Healthcare, Buckinghamshire, England*) for 5 minutes and then developed with a camera system (Fluorchem SP; *Alpha Innotech, San Leandro, CA*)

Quantification

Densitometry software (AlphaEaseFC V.4.1.0; *Alpha Innotech, San Leandro, CA*) will be used to quantify blot brightness. Each blot will be measured three times with the average number being used.

Statistics

A multivariate analysis of variance (MANOVA) will be used to determine significance. If significance is found, then Tukey's post hoc will be used as a follow up test. Significance will be set at $P<.05$.

CHAPTER 4

Results

Male Wistar rats were heat-shocked in a water bath until their internal temperature had reached 41.0-41.5°C for 20 minutes. 48 hours later the rats ran for 5 min, followed by 2 min rest, at 18m/min on a 16° downhill treadmill for a total protocol length of 126 minutes. SOL, VLR and VLW muscles were harvested at 2hr and 48hr post-exercise. Through SDS-PAGE followed by Western immunoblotting, there was no significant difference between total integrin expression measured by the beta1 subunit, total and phosphorylated ERK1/2, total and phosphorylated c-Src and the ratio of phosphorylated to total ERK1/2 and c-Src in any of the treament groups, CON, EE or HS+EE, in any muscle, SOL, VLR or VLW, at any of the time points. These outcomes demonstrate that a downhill, eccentric exercise protocol is not sufficient to alter IN signaling in heat shocked or eccentrically exercised rat skeletal muscle versus controls.

Descriptions of Figures

Figure 4.1: Beta1 Subunit in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.980)* and HS+EE (*p=.966)* and EE vs HS+EE (*p=.997)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.947)* and HS+EE (*p=.998)* and EE vs HS+EE (*p=.938)*].

Figure 4.2: Beta1 Subunit in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.962)* and HS+EE (*p=.868)* and EE vs HS+EE (*p=.576)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.407)* and HS+EE (*p=.576)* and EE vs HS+EE (*p=.915)*].

Figure 4.3: Beta1 Subunit in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.877)* and HS+EE (*p=.709)* and EE vs HS+EE (*p=.909)*]. *B)* No significant difference in 48hr VLW [CON vs EE (*p=.304)* and HS+EE (*p=.289)* and EE vs HS+EE (*p=.999)*].

Figure 4.4: Total ERK1/2 in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.935)* and HS+EE (*p=.999)* and EE vs HS+EE (*p=.853)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.342)* and HS+EE (*p=.447)* and EE vs HS+EE (*p=.959)*].

Figure 4.5: Total ERK1/2 in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.978)* and HS+EE (*p=.855)* and EE vs HS+EE (*p=.894)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.913)* and HS+EE (*p=.389)* and EE vs HS+EE (*p=.427)*].

Figure 4.6: Total ERK1/2 in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.217)* and HS+EE (*p=.403)* and EE vs HS+EE (*p=.403)*]. *B)* No significant differences in 48hr VLW [CON vs EE (*p=.645)* and HS+EE (*p=.713)* and EE vs HS+EE (*p=.427)*].

Figure 4.7: Phosphorylated ERK1/2 in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.121)* and HS+EE (*p=.183)* and EE vs HS+EE (*p=.942)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.334)* and HS+EE (*p=.928)* and EE vs HS+EE (*p=.325)*].

Figure 4.8: Phosphorylated ERK1/2 in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.413)* and HS+EE (*p=.481)* and EE vs HS+EE (*p=.985)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.459)* and HS+EE (*p=.996)* and EE vs HS+EE (*p=.468)*].

Figure 4.9: Phosphorylated ERK1/2 in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.824)* and HS+EE (*p=.900)* and EE vs HS+EE (*p=.976)*]. *B)* No significant differences in 48hr VLW [CON vs EE (*p=.543)* and HS+EE (*p=.981)* and EE vs HS+EE (*p=.468)*].

Figure 4.10: Ratio of Phosphorylated to Total ERK1/2 in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.084)* and HS+EE (*p=.179)* and EE vs HS+EE (*p=.831)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.376)* and HS+EE (*p=.917)* and EE vs HS+EE (*p=.402)*].

Figure 4.11: Ratio of Phosphorylated to Total ERK1/2 in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.477)* and HS+EE (*p=.704)* and EE vs HS+EE (*p=.866)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.533)* and HS+EE (*p=.903)* and EE vs HS+EE (*p=.653)*].

Figure 4.12: Ratio of Phosphorylated to Total ERK1/2 in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.956)* and HS+EE (*p=.832)* and EE vs HS+EE (*p=.917)*]. *B)* No significant differences in 48hr VLW [CON vs EE (*p=.373)* and HS+EE (*p=.886)* and EE vs HS+EE (*p=.451)*].

Figure 4.13: Total c-Src in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.919)* and HS+EE (*p=.884)* and EE vs HS+EE (*p=.484)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.714)* and HS+EE (*p=.881)* and EE vs HS+EE (*p=.924)*].

Figure 4.14: Total c-Src in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.477)* and HS+EE (*p=.125)* and EE vs HS+EE (*p=.090)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.405)* and HS+EE (*p=.116)* and EE vs HS+EE (*p=.571)*].

Figure 4.15: Total c-Src in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.950)* and HS+EE (*p=.964)* and EE vs HS+EE (*p=.998)*]. *B)* No significant differences in 48hr VLW [CON vs EE (*p=.103)* and HS+EE (*p=.312)* and EE vs HS+EE (*p=.665)*].

Figure 4.16: Phosphorylated c-Src 527 in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.622)* and HS+EE (*p=.996)* and EE vs HS+EE (*p=.366)*]. *B)* No significant differences in 48hr Sol [CON vs EE (*p=.995)* and HS+EE (*p=.973)* and EE vs HS+EE (*p=.920)*].

Figure 4.17: Phosphorylated c-Src 527 in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.157)* and HS+EE (*p=.540)* and EE vs HS+EE (*p=.481)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.755)* and HS+EE (*p=.940)* and EE vs HS+EE (*p=.884)*].

Figure 4.18: Phosphorylated c-Src 527 in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.335)* and HS+EE (*p=.241)* and EE vs HS+EE (*p=.951)*]. *B)* No significant differences in 48hr VLW [CON vs EE (*p=.179)* and HS+EE (*p=.438)* and EE vs HS+EE (*p=.720)*].

Figure 4.19: Ratio of Phosphorylated to Total c-Src in SOL. *A)* No significant differences between treatments in 2hr Sol [CON vs EE (*p=.996)* and HS+EE (*p=1.000)* and EE vs HS+EE (*p=.997)*]. *B0* No significant differences in 48hr Sol [CON vs EE (*p=.434)* and HS+EE (*p=.420)* and EE vs HS+EE (*p=.999)*].

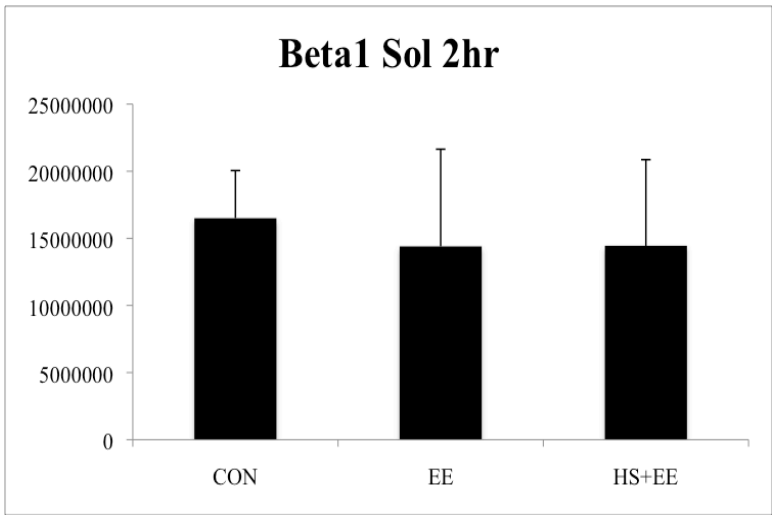
Figure 4.20: Ratio of Phosphorylated to Total c-Src in VLR. *A)* No significant differences between treatments in 2hr VLR [CON vs EE (*p=.705)* and HS+EE (*p=.496)* and EE vs HS+EE (*p=.902)*]. *B)* No significant differences in 48hr VLR [CON vs EE (*p=.548)* and HS+EE (*p=.304)* and EE vs HS+EE (*p=.832)*].

Figure 4.21: Ratio of Phosphorylated to Total c-Src in VLW. *A)* No significant differences between treatments in 2hr VLW [CON vs EE (*p=.575)* and HS+EE (*p=.679)* and EE vs HS+EE (*p=.975)*]. *B)* There were no significant differences in 48hr VLW [CON vs EE (*p=.996)* and HS+EE (*p=.969)* and EE vs HS+EE (*p=.918)*].

Figures

Figure 4.1: Total Beta1 Subunit SOL

A



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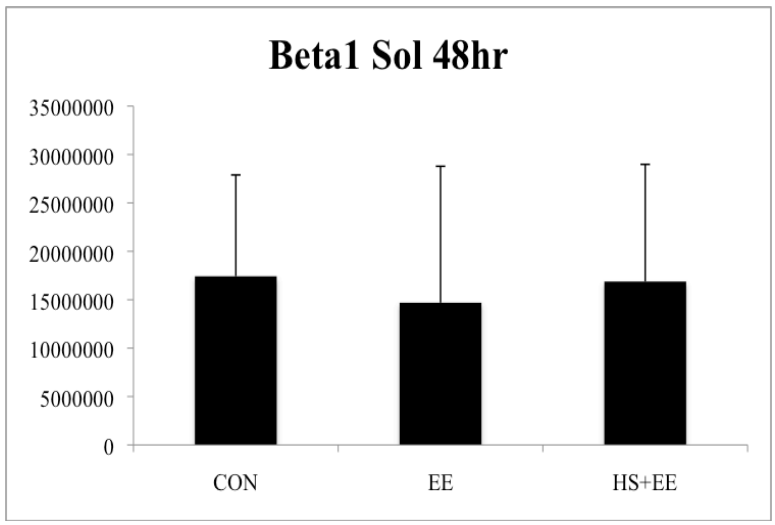

Figure 4.2: Total Beta1 Subunit VLR

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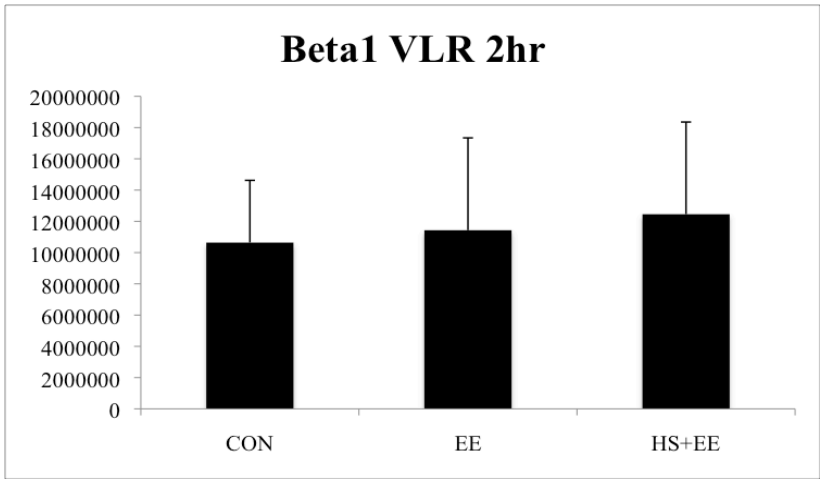

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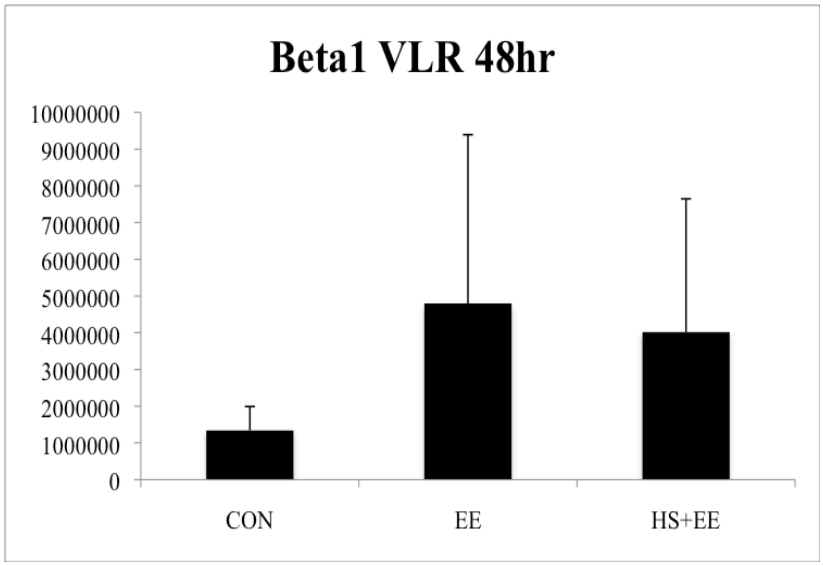

Figure 3: Total Beta1 Subunit VLW

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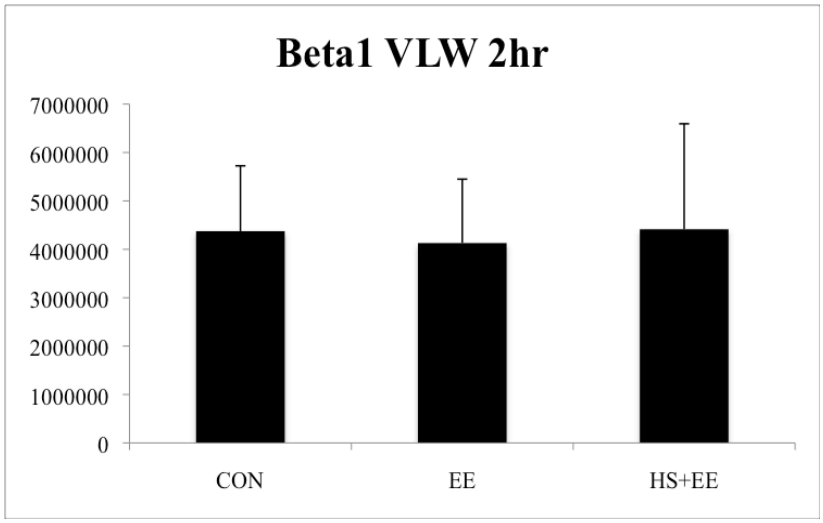

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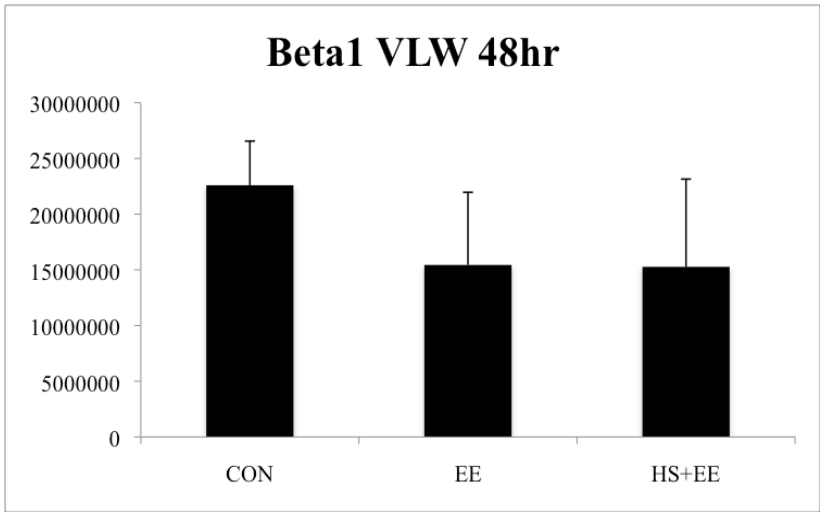

Figure 4: Total ERK1/2 SOL

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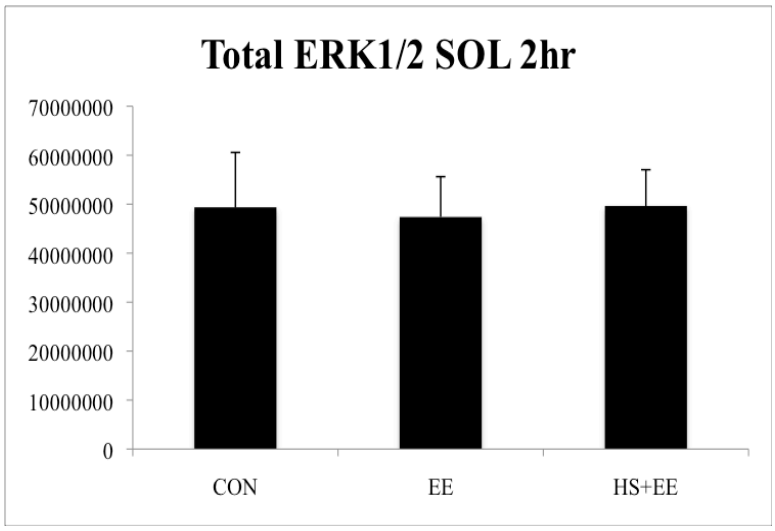

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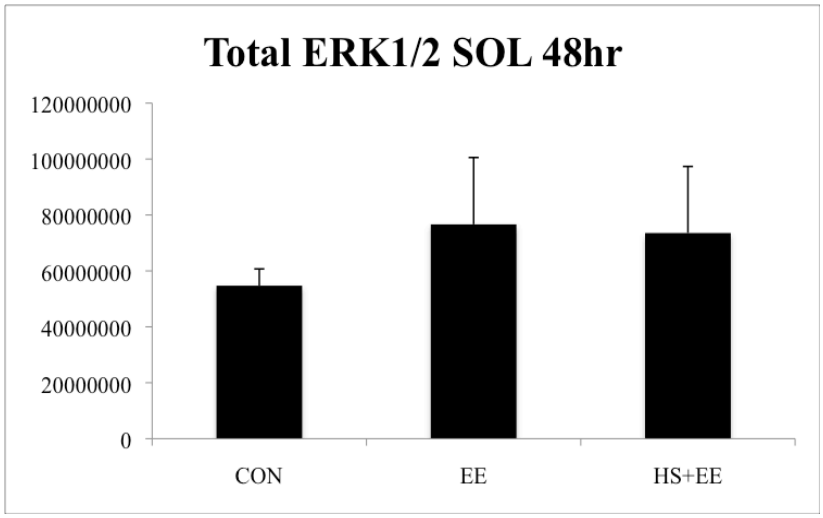

Figure 5: Total ERK1/2 VLR

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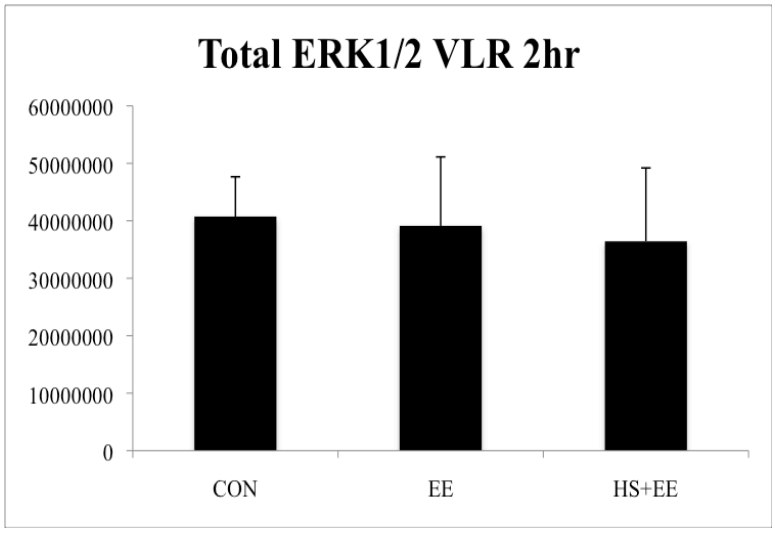

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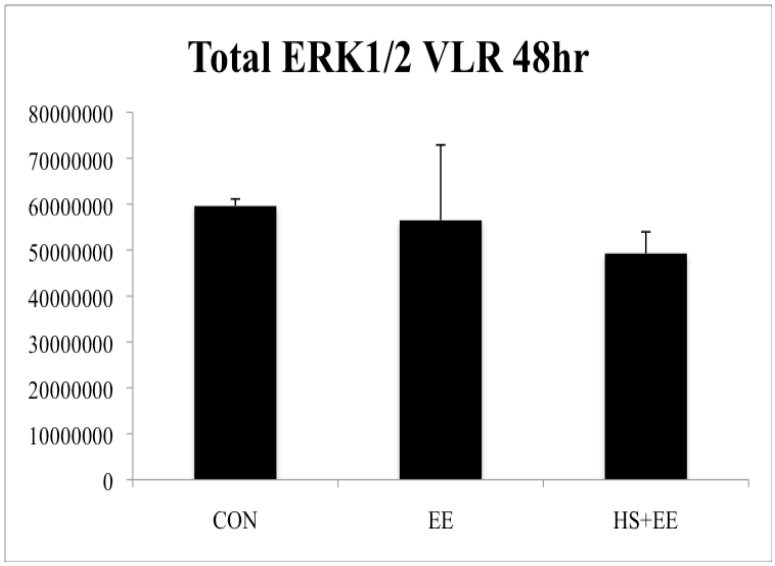

Figure 6: Total ERK1/2 VLW

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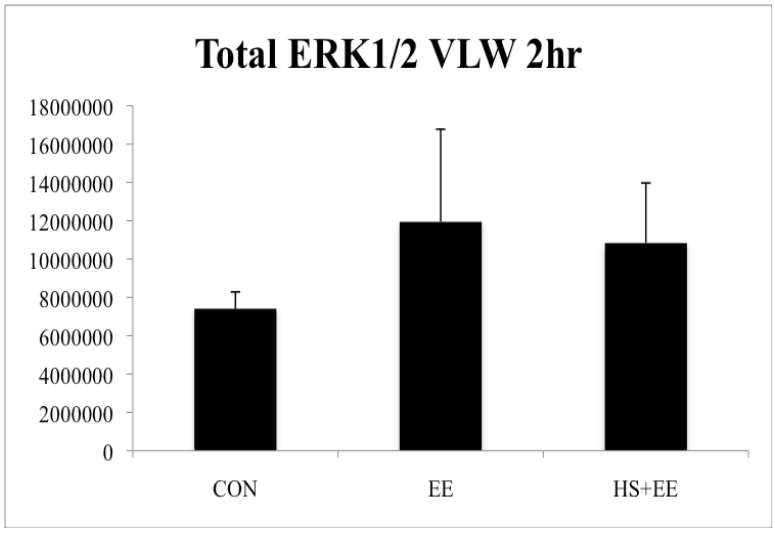

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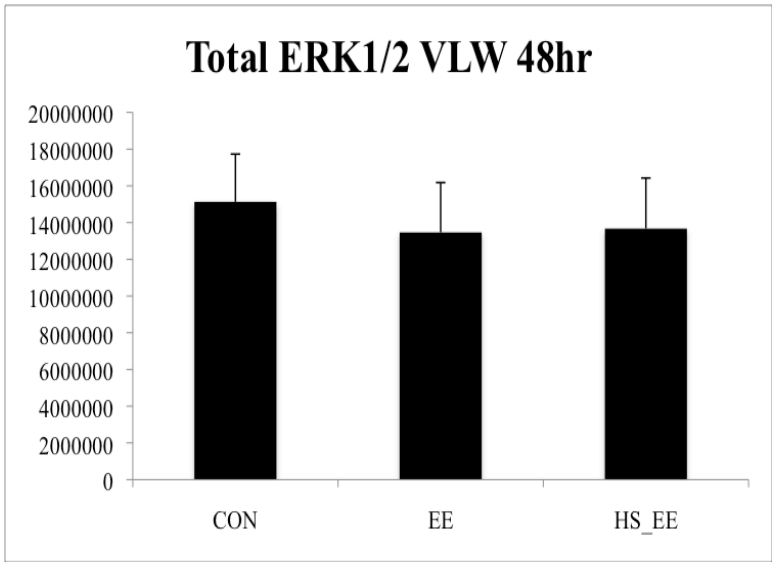

Figure 7: pERK1/2 Sol

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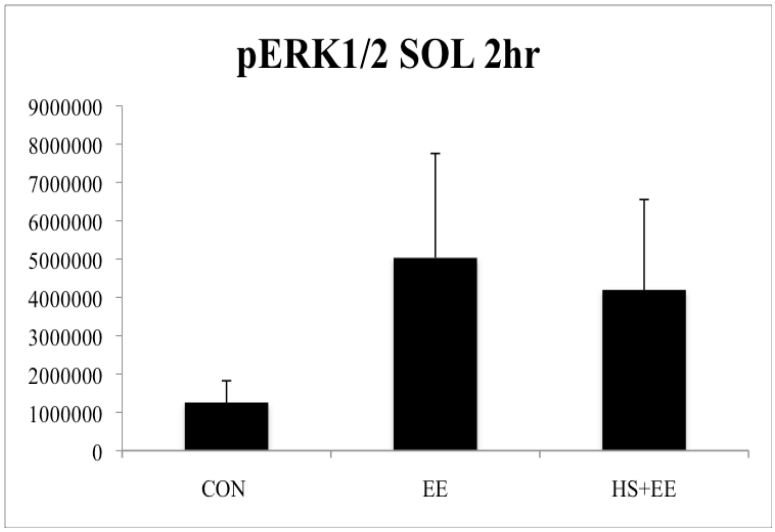

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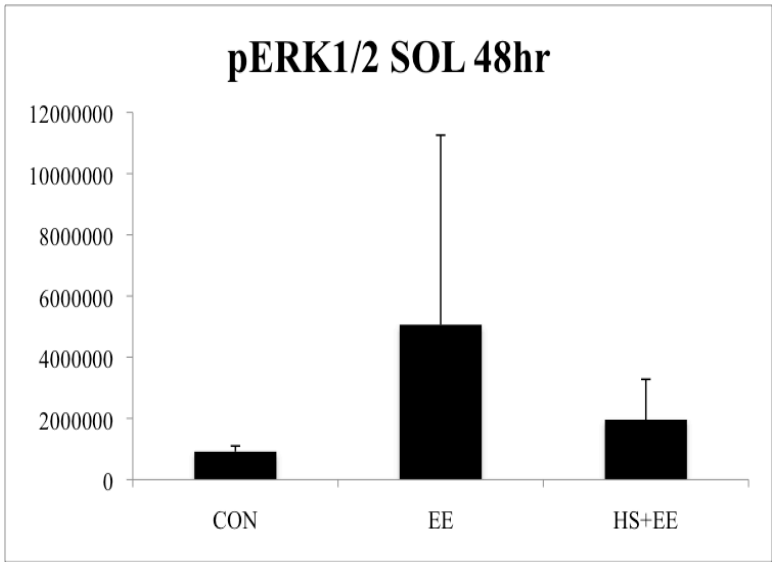

Figure 8: pERK1/2 VLR

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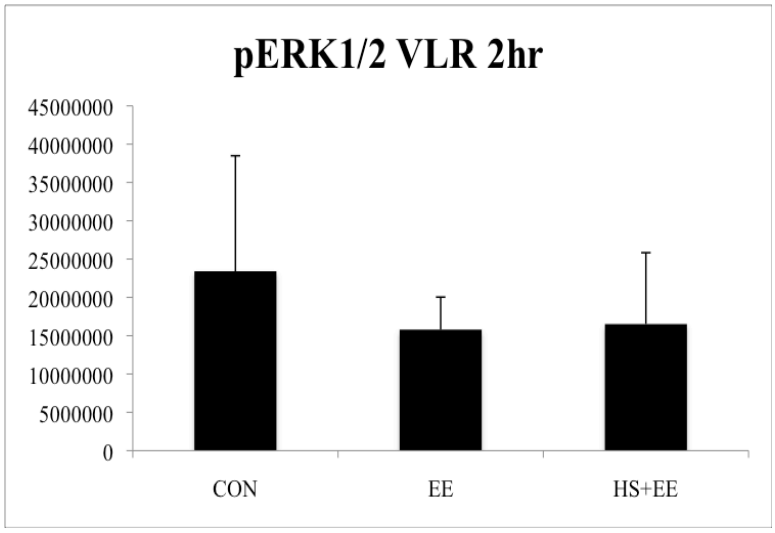

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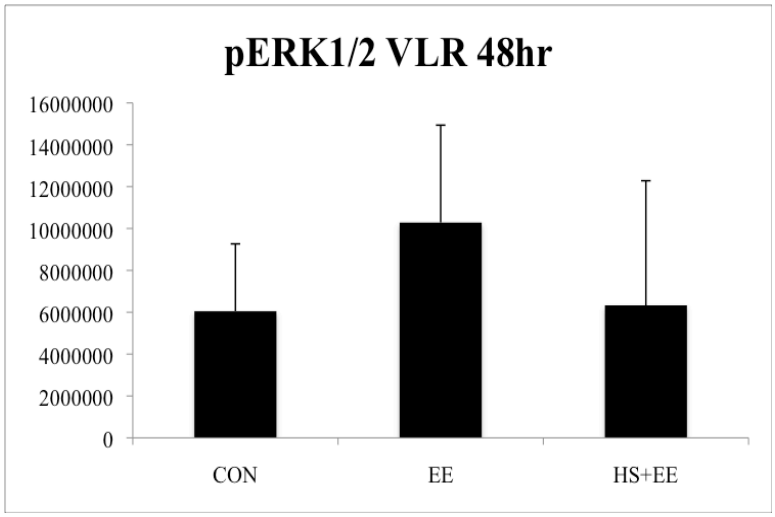

Figure 9: pERK1/2 VLW

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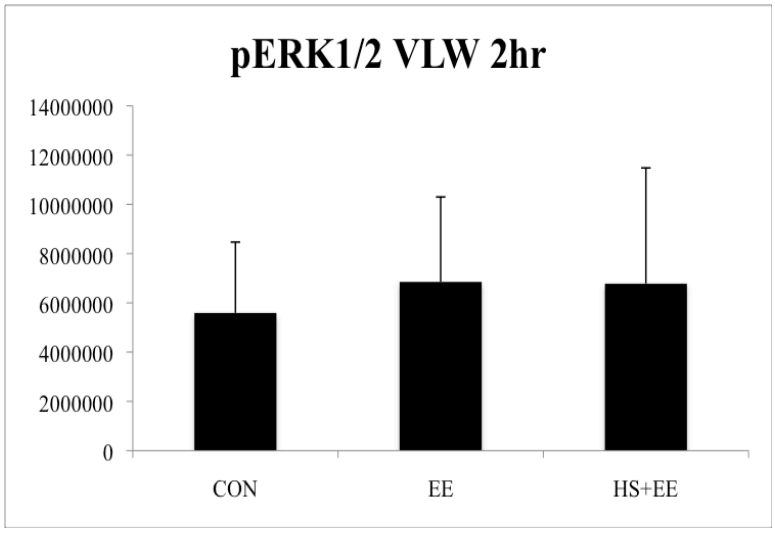

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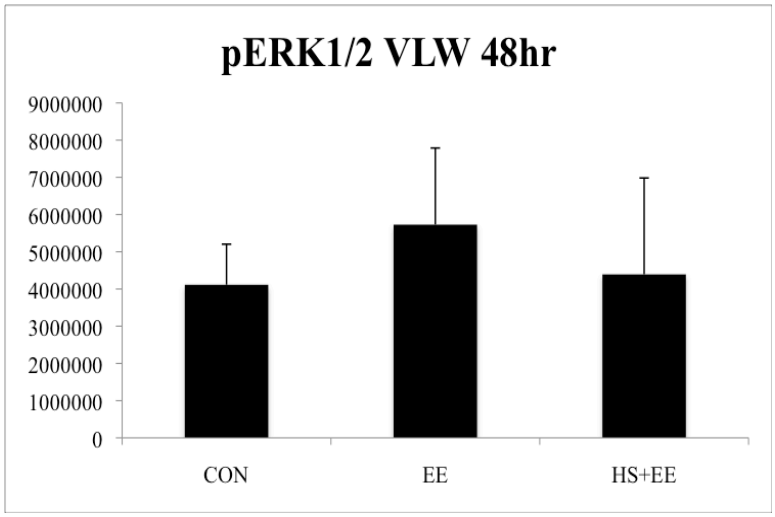
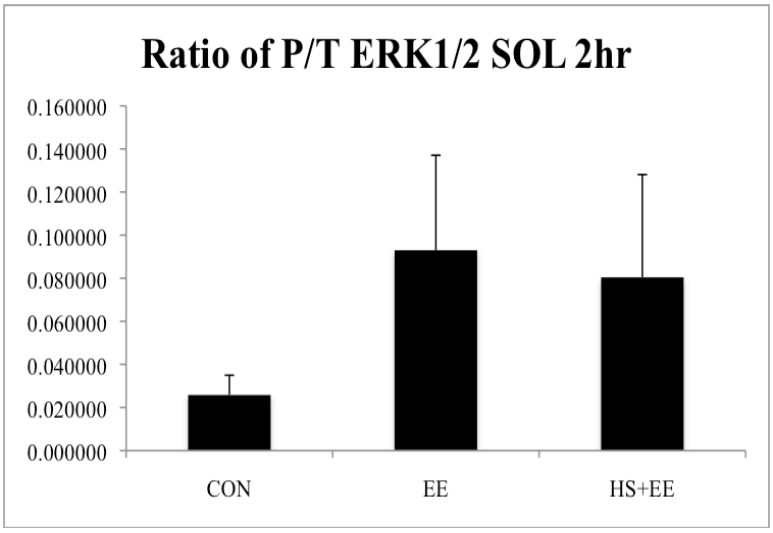


Figure 10: Ratio of p/t ERK1/2 SOL

A



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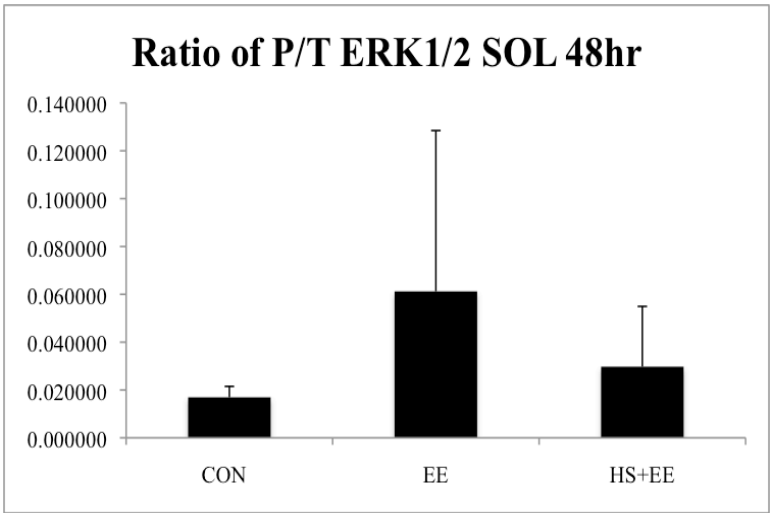

Figure 11: Ratio of p/t ERK1/2 VLR

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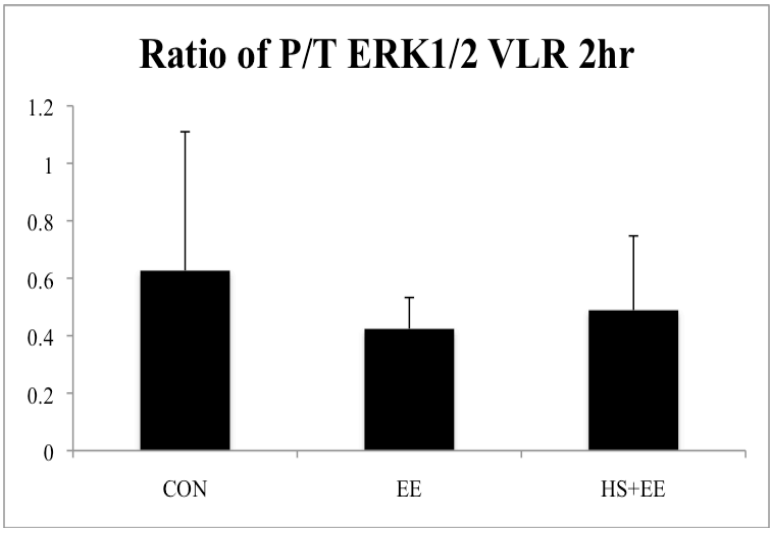

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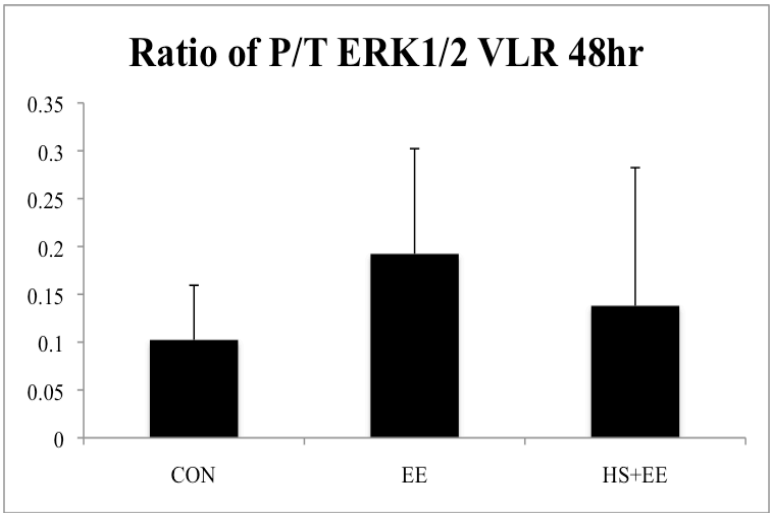

Figure 12: Ratio of p/t ERK1/2 VLW

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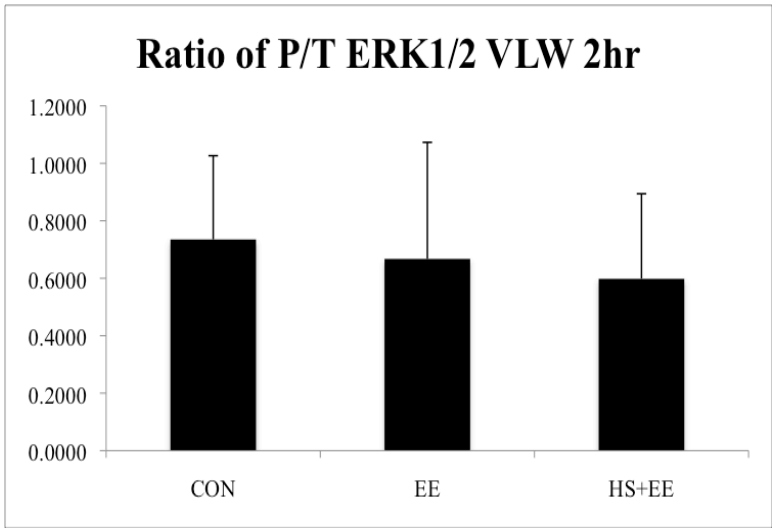

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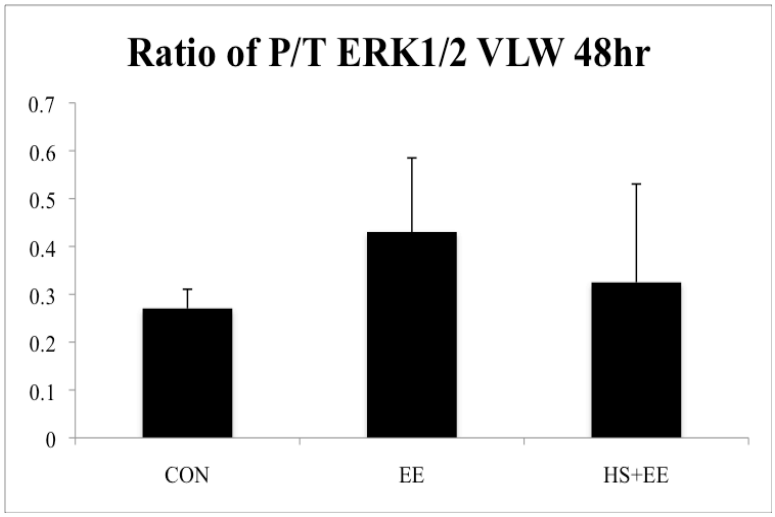

Figure 13: Total c-Src Sol 2hr

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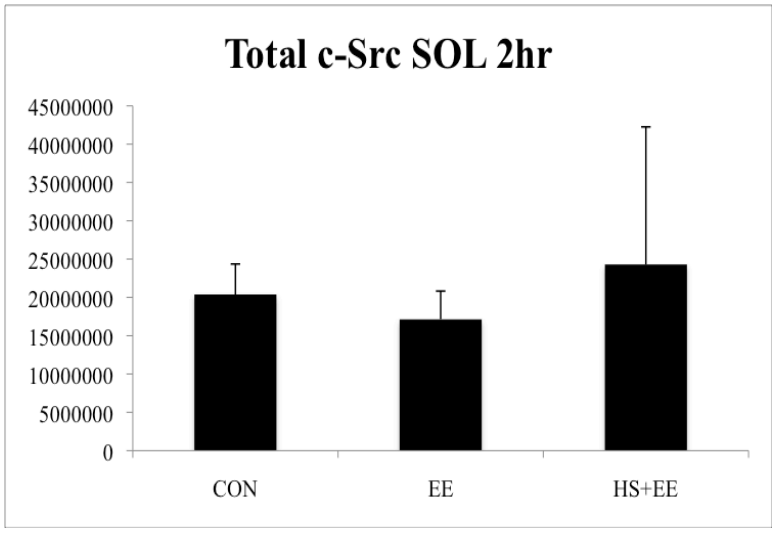

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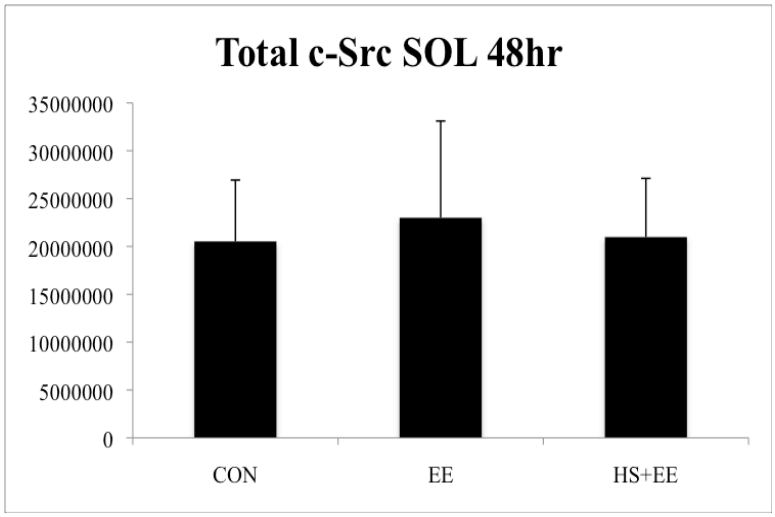

Figure 14: Total c-Src VLR

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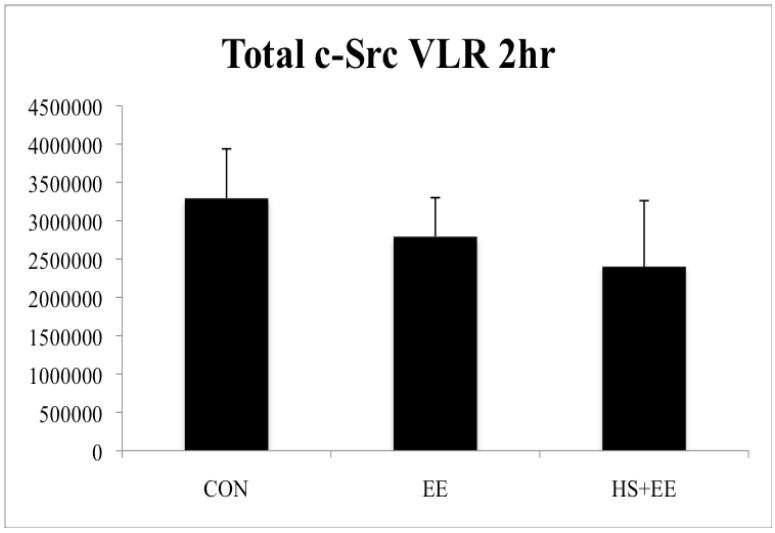

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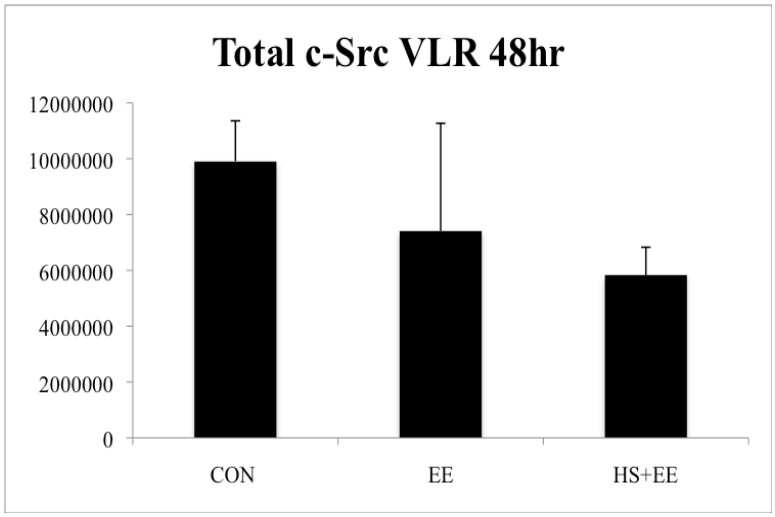

Figure 15: Total c-Src VLW

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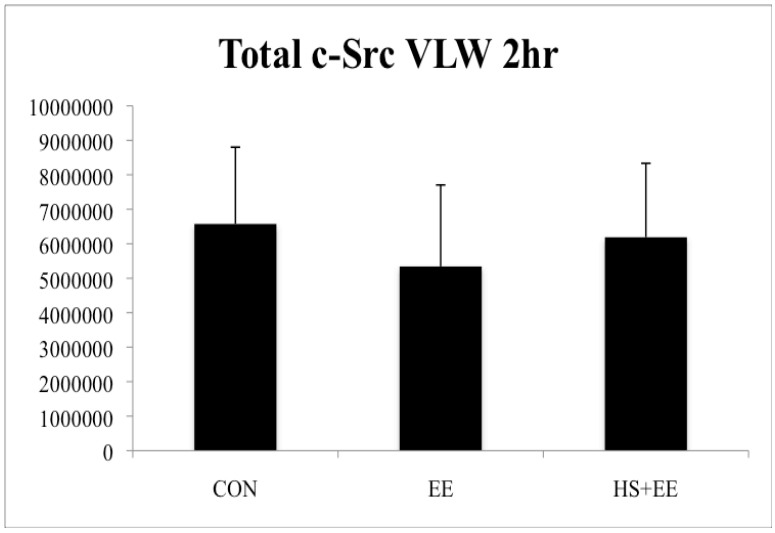

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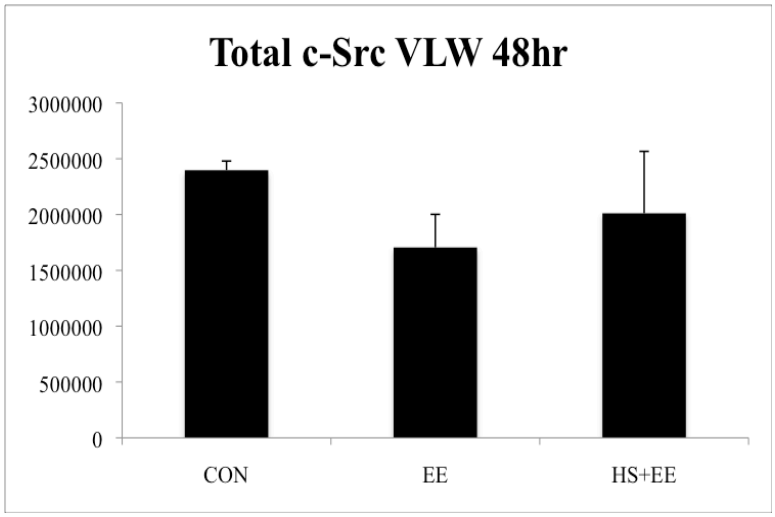

Figure 16: pc-Src 527 Sol

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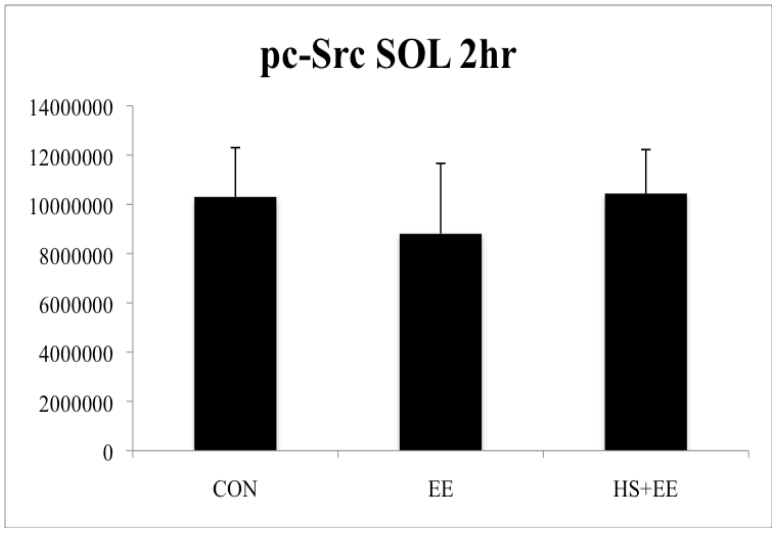

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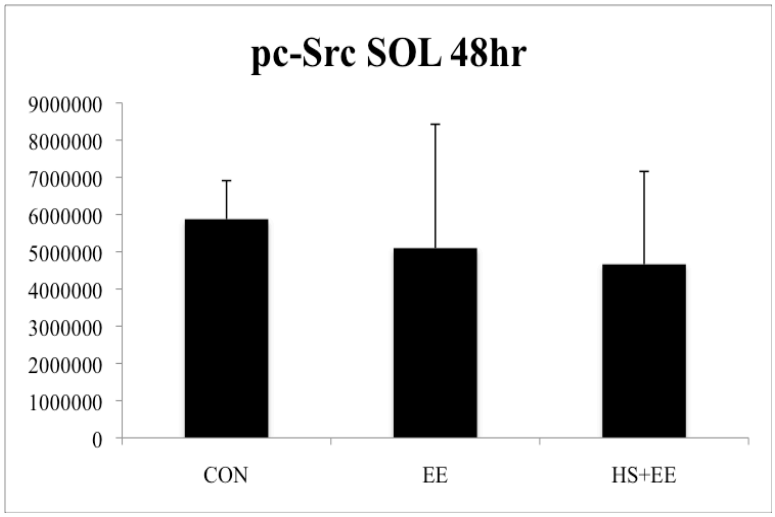

Figure 17: pc-Src 527 VLR

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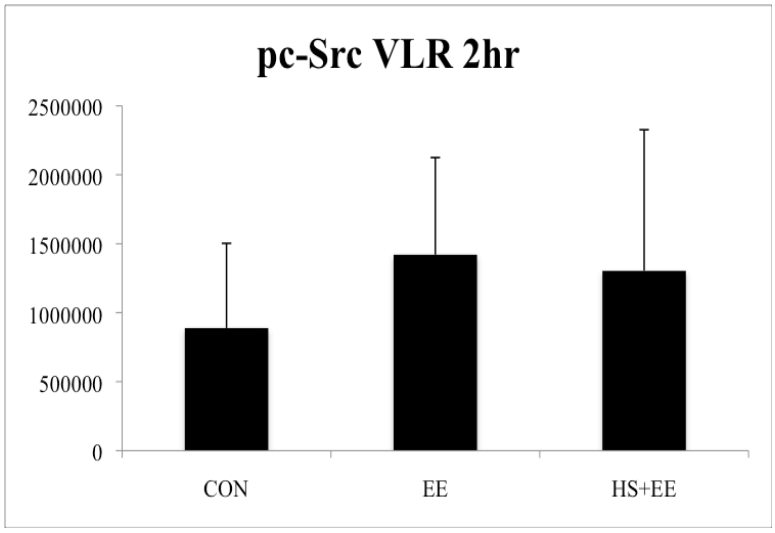

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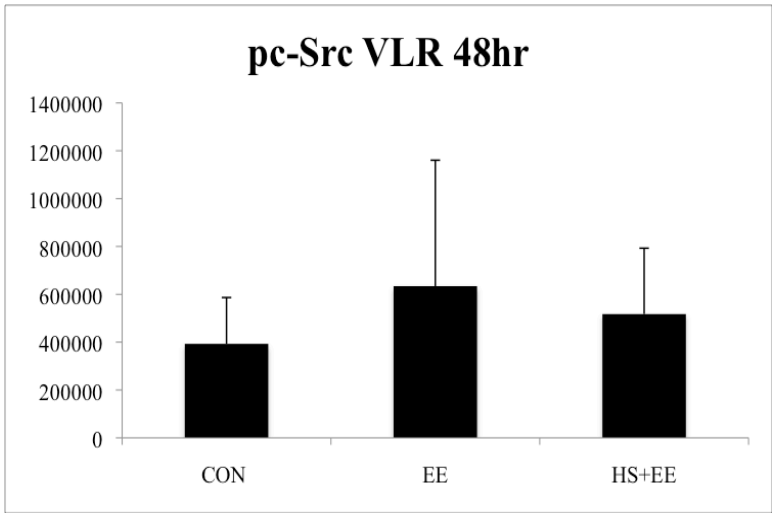

Figure 18: pc-Src 527 VLW

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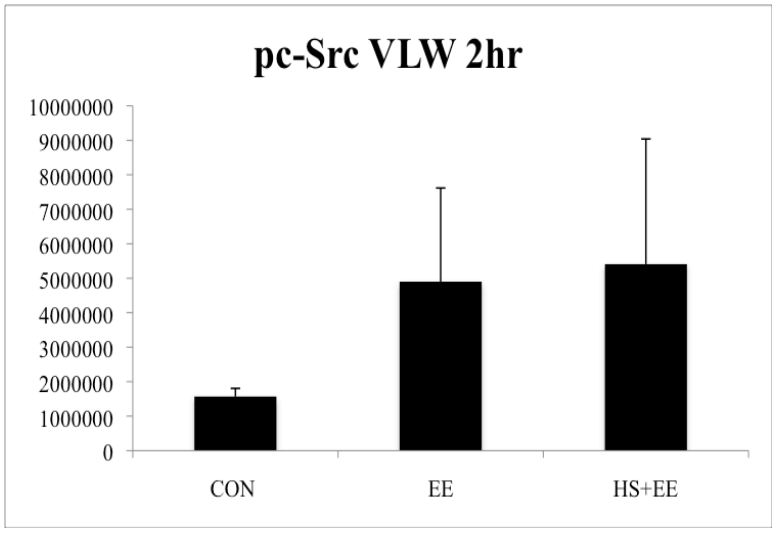

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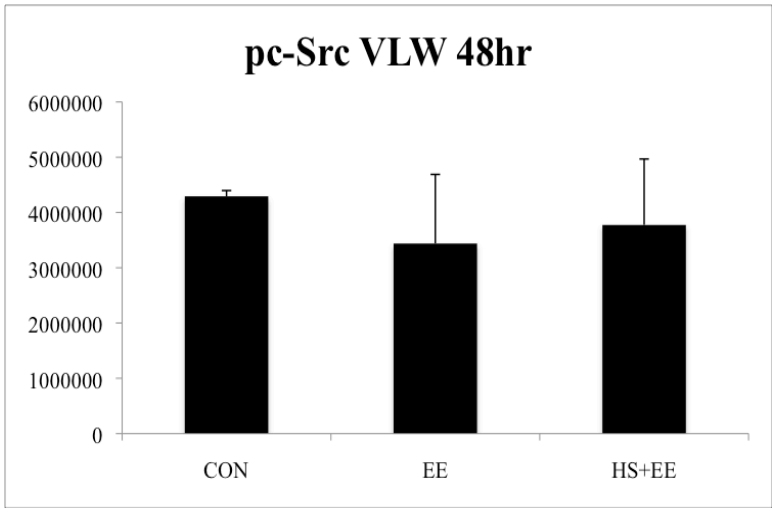

Figure 19: Ratio of p/t c-Src Sol

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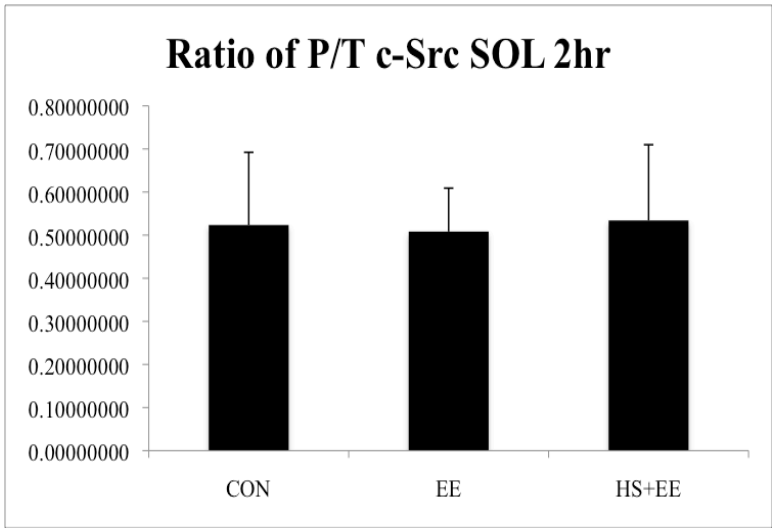

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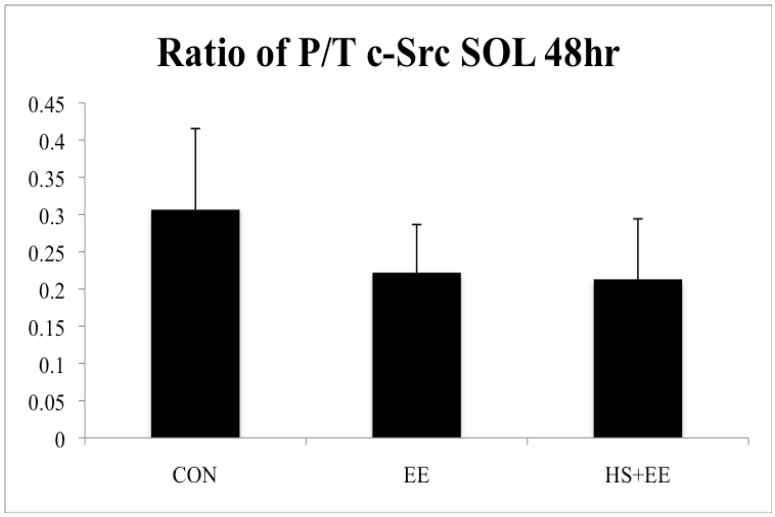

Figure 20: Ratio of p/t c-Src VLR

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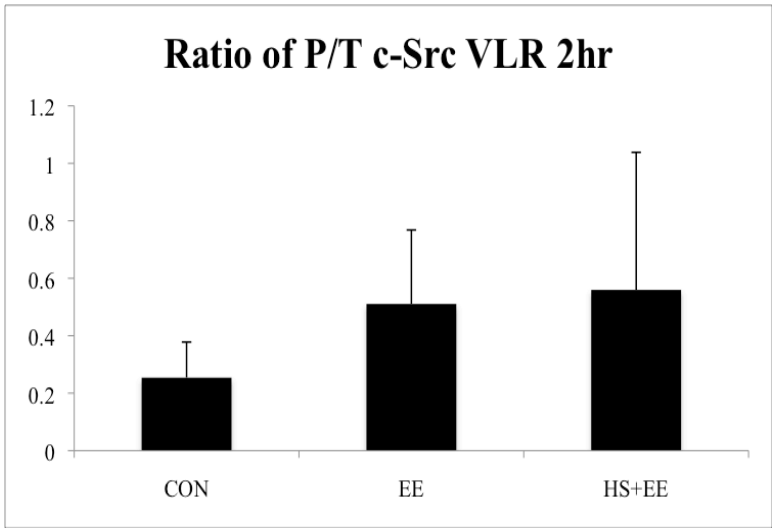

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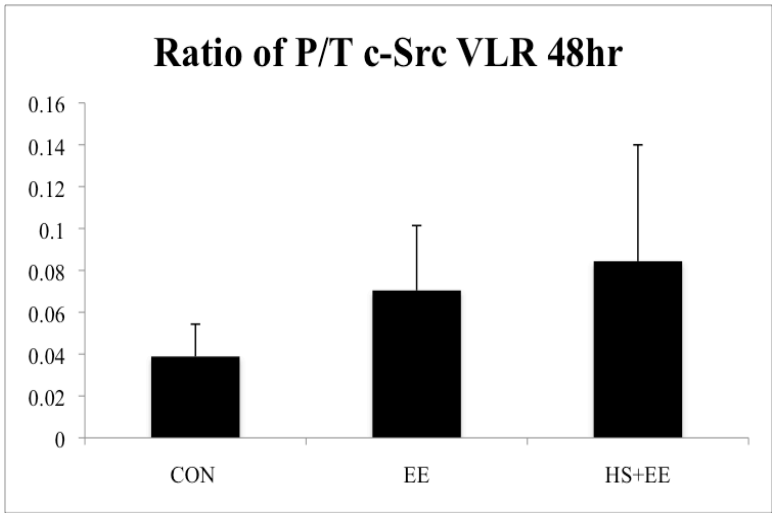

Figure 21: Ratio of p/t c-Src VLW

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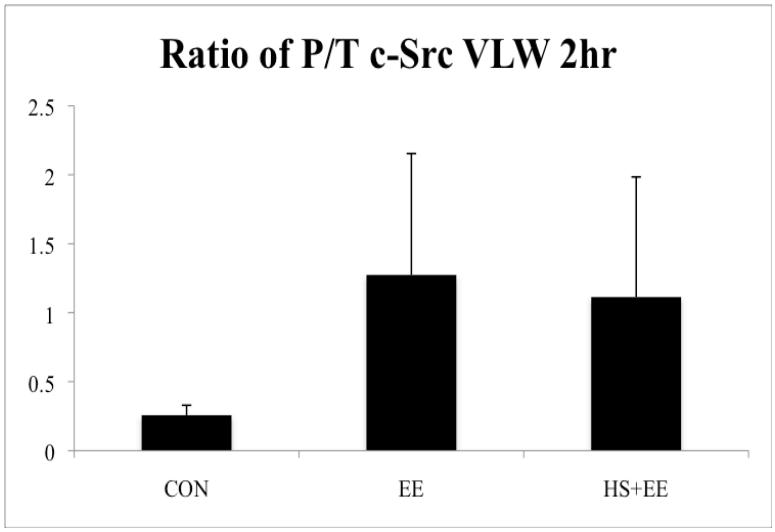

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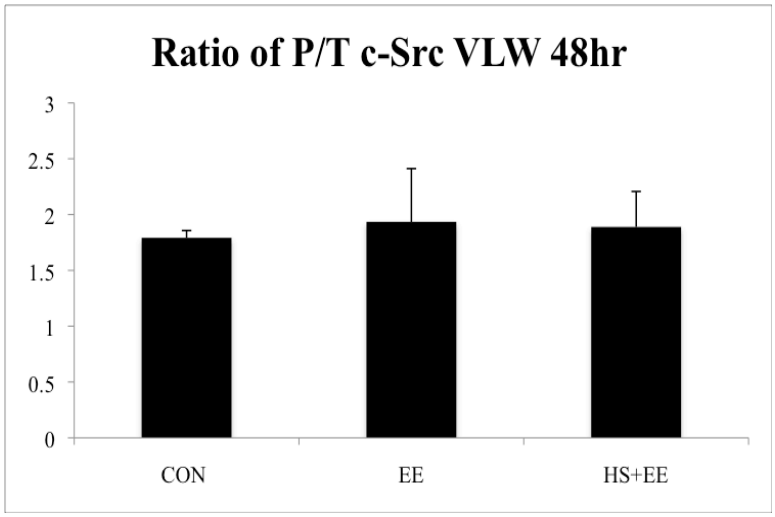

Chapter 5

Discussion

There have been many experiments that have dealt with IN in various cells and conditions yet there is a lack of literature that deals with IN and SM, especially in healthy muscle cells. This current study aimed to look at IN signaling in healthy rat SM after a a bout of eccentric exercise. What our data demonstrates is a two hour eccentric exercise protocol, regardless of heat treatment, does not significantly alter IN levels or IN signaling in rat SOL, VLR and VLW muscles at 2hr and 48hr post exercise. While there are not a lot of studies dealing with IN expression in SM without pathological conditions, there are a few similar studies that can adequately compare with the current study. The outcomes of the current study contrast to a similar study carried out by Boppart, et al., (10). They ran female mice downhill at a slightly slower speed (17m/min) and at a more severe grade (20°) compared to our study as well as exercising the mice for only 30 minutes. They saw a significant 70% increase in the expression of alpha7 subunit at 24 hours post-exercise in the gastrocnemius and soleus complex and an elevated, although not significant, alpha7 level 1 week after the exercise protocol. Since this study used mice, there may be the possibility that mice respond to eccentric contractions more readily than rats. There is also the prospect that there could be a gender difference. Boppart and colleagues used females while this study used all males. The duration of the exercise may have been a factor as well. They report that they ran their mice for 30 min straight while the current study ran rats repeatedly for 5 minutes followed by a 2 minute break. This break may be enough time to allow IN signaling to return to baseline although no study could be found that looked at IN and downstream signaling over time.

Boppart and colleagues also used this protocol several years later to look at mRNA expression of the alpha7 subunit (11). This time they found a 5.4 fold increase 3 hours post exercise. The levels returned to normal 24 hrs and 1 week post exercise. This compliments the previously described study. If the mRNA is increased drastically it may lead to the increase in protein expression seen 24 hrs and 1 week after exercise. Kääriäinen and colleagues (44) incited muscle damage by completely transecting SOL unilaterally and measuring recovery and alpha7beta1 expression over a span of 56 days. What they reported was an increase in the beta1variant beta1A, the variant most apparent in developing or recovering muscle, in stained regenerating muscle fibers at 2 days and an increase in appearance of the overall beta1 subunit by staining at the site of injury at 3 days. So while this may not fit perfectly in line with the current study is does show in increase in IN presence during recovery. It is also possible that the transection is far more traumatic to the cell than a downhill exercise protocol and may induce a reaction that is larger in magnitude.

We also know that IN are a major player in mechanotransduction. It has been shown that patients with Duchenne's muscular dystrophy have an elevated level of alpha7beta1 integrin using quantified staining and RT-PCR designed to detect alpha7 subunits (37). This upregulation of alpha7beta1 IN is obviously there to provide a backup mechanism for cellular protection and to anchor the cell during a muscular contraction. A lack of the alpha7 subunit can be also be lethal in murines. Welser and colleagues demonstrated that mice bred to lack only the alpha7 gene had a 54.3% death rate *in utero*, weighed 60% less

than wild type mice and had a 20% loss of strength compared to wild type mice (109). This again shows that IN are necessary for the transmission of force and cell survival.

There have been no studies located that have looked at c-Src in SM *in vivo*, let alone exercise and SM. To try and understand how exercise may activate c-Src in SM we can try to look at FAK. The only study found concerning FAK and exercise used a resistance training protocol. It was shown that doing 4 sets of 10 reps of isolateral leg extensions and leg press did not increase FAK activation (29). We can then hypothesize that this exercise protocol is not sufficient to activate c-Src. With the lack of information about c-Src in SM, we must look to other cells such as cardiac tissue. Kuppuswamy, et al., (54) used a pressure overloaded cat right ventricle and saw an increase in total c-Src at 4hr, a peak at 48hr and a return to baseline after 1 week. This makes it somewhat surprising that we saw no difference in our c-Src totals, especially at the 48hr timepoint. Again, this points to the possibility that the exercise protocol was not strenuous enough. No study can be located that describes how quickly c-Src becomes activated through FAK. But it should be noted that when placing angiotensin II (a c-Src activator) in cultured vascular smooth muscle cells and measuring the time points of c-Src activity, there was a 3 fold increase in c-Src activity within 3 minutes and then a gradual decline to 2 fold at 10 minutes (41). This again points to the fact that we may have missed the optimal time point. It is also interesting that we did not see an increase in c-Src because it seems to be activated by stretch through a pathway other than FAK. It was shown that stretching of C2C12 cells increased c-Src in the dystroglycan complex in an IN dependent manner and that inhibition of c-Src by the inhibitor PP2 stopped cell proliferation by 5 fold (115).

It was also surprising that there was no increase in ERK1/2 activation. Martineau and Gardiner (65) have shown that EE has the largest impact in activating MAPK's in rat skeletal muscle when compared to isometric and concentric By hooking rat SM into a muscle stimulator they found a five-fold increase in pERK1/2 compared to a control. They also found a three-fold increase in concentric contractions, making it even stranger that a 2hr eccentric exercise protocol did not result in an increase in ERK1/2 activation for the current study. It has been shown that an acute bout of endurance exercise can activate ERK1/2 in human skeletal muscle. Untrained but healthy men cycled at 70% VO2max for one hour and saw a dramatic increase in ERK1/2 activation immediately after exercise (7). However, levels of pERK1/2 had returned to basal levels after three hours of recovery. This shows that we may have missed the optimal time for seeing the elevated activation of ERK1/2. We also may not have activated IN sufficiently enough to start the cascade. In NRVM that had slightly dysfunctional beta1 subunits, there was decrease in activated FAK and ERK1/2 in stretched cells compared to control cells (55). What this demonstrates is that a functional beta1 signal is necessary for ERK1/2 phosphorylation. This study also found that when the cell had high levels of FRNK, a FAK inhibitor, then pERK1/2 levels go down, again showing that for full ERK1/2 activation a valid IN related signal is necessary. Takeishi, et al., (95) have shown a 6 fold increase of ERK1/2 activity at 10 min post cardiac overload in guinea pig hearts. This peak activation then decreased 33% at 1day post. Again, this is evidence that shows that we should have seen activation of ERK1/2.

It has already been stated that small heat shock proteins have been able to activate FAK (56, 108). A limiting factor of this current study is that there was no analysis of FAK to determine if in the HS group there was an increase in FAK expression or activation comparatively. It has recently been shown that HS may activate alpha3beta1 IN. In breast carcinoma cell lines, researchers demonstrated that Hsp60 immunoprecipitates with alpha3beta1 IN (5). They also showed that in cells with recombinant Hsp60, the cell with the alpha3 subunit demonstrated increased motility compared to an alpha2 subunit. This shows that Hsp60 can activate IN with a beta1 subunit but is dependent on the alpha subunit. It has also been shown that Hsp 60 can create bridges with fibronectin, fibronection binding protein, and beta1 subunits in endothelial cells (23) ultimately causing the activation of the IN in epithelial cells. Since we only looked at total beta1 subunit, it is quite possible that there may be an increase in activated IN in the HS group because of the relationship with Hsp60. Hsp may play a role downstream of IN. In cervical cancer cells, the inhibition of Hsp90 attenuates activation of FAK and hinders tumor growth (92) giving Hsp90 a role of, perhaps, protecting FAK or IN signaling. Again, we would need to look at FAK activation to see how this interaction with Hsp90 could potentially affect our samples. Even further down the pathway, elevating heat in NIH3T3 cells to 43°C for 10 minutes increased activation of c-Src by a little over 2 fold after 5 minutes and maintained elevated levels for 15 minutes (60). The study also showed an increase in ERK1/2 activity. Lastly, ERK1/2 activation was increased 60 fold in heat shocked interleukin 3 cells (74) at 60 min and 120 min. So, again, it is very suprising that we saw no increase in downstream of IN singaling in all aspects of our study.

Limitations

An apparent limitation of this study was the lack of focus on FAK. Considering that Hsp and IN can activate it, to get a clarified view of the complete IN signaling pathway, looking at FAK is a must. Also, trying to determine activation of IN was a limiting factor of this study. It would provide a glimpse into determining if our protocol is sufficient enough to cause IN activation and, in turn, determine what exercise and load may be necessary to activate these proteins.

Sample size was also a limiting factor. Because of our small sample sizes, we had very large variabilities in our densitometry values, possibley making it more difficult to find an accurate and possibly significant outcome.

Our time points may have been a limiting factor. It has been seen that ERK1/2 has been activated at the time points we covered but c-Src seems to become active very quickly and may not have had enough activity at the 2hr and 48hr post-exercise timepoints.

It may also be possible that our exercise protocol may not have been sufficient to activate these proteins. The study that found increases in alpha7 expression (10) used a more aggressive slope for a continuous amount of time while our study was a two hour protocol.

Future Research

There is still plenty of information that needs to be resolved with IN signaling in SM. First, it needs to be known what the appropriate magnitude of muscular contraction is

needed to reliably and repeatedly activate IN. It also needs to be determined what is the optimal timing of all of these IN related proteins. If we can accurately predict when FAK is activated we may better understand its role in the pathways that it controls and how specific these pathways are in regards to contraction and HS. It also needs to be determined if HS plays a major role in SM through FAK. Studies could possibly look into using pharmacological inhibitors to determine which pathways HS and FAK may go through. The same may be determined for c-Src and ERK1/2.

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APPENDIX A

Raw data for alpha7beta1 Integrin

2hr

SOL: (***CON***=3: 15527485±4421965; ***EE***; n=8: 14609996±7711416; ***HS+EE***: n=8; 13531410±6201014)
VLR: (***CON***=3; 10563617±4836214; ***EE***=8; 11661395±6276239; ***HS+EE***=8; 12671899±6270266)
VLW: **(*CON*** =3; 3679673±729936; ***EE***=8; 4257895±1350019; ***HS+EE***=8; 4622543±2231892)

Significance Levels for 2hr

CON compared to EE (*SOL: p=.980; VLR: p=.962; VLW: p=.877*) and HS+EE (*SOL*: *p=.966*; *VLR: p=.868*; *VLW*: *p=.709*) and EE compared to HS+EE (*SOL: p=.997; VLR: p=.942; VLW: p=.909*).

48hr

SOL: (***CON***=3; 20353252±10467104; ***EE***:n=8; 14688935±14096905; ***HS+EE***: n=8; 16881546±12097800)
VLR: ***(CON***=3; 1337774±653482; ***EE***=8; 4797242±4596025; ***HS+EE***=8; 4016059±3632115)
VLW: (***CON***=3; 22594159±3965464; ***EE***=8; 15440392±6523425; ***HS+EE***=8; 15275500±7872437) in

Significance levels for 48hr

CON compared to EE (*SOL: p=.947; VLR: p=.407; VLW: p=.304)* and HS+EE (*SOL: p=.998; VLR: p=.576; VLW: p=.289*) and EE compared to HS+EE (*SOL: p=.938; VLR: p=.915; VLW: p=.999*).

Raw data for total ERK1/2

2hr

SOL: (***CON***=3; 49370528±11201938; ***EE***=8; 47391828±;8230230; ***HS+EE***=8; 49642664±7401158)
VLR: (***CON***=3; 40735017±6916230; ***EE***=8; 39115421±11999886; ***HS+EE***=8; 36429977±12768147)

VLW" (***CON***=3; 7408697±875360; ***EE***=8; 11944792±4834850; ***HS+EE***=8; 10837911±3136231)

Significance levels for 2hr

CON compared to EE (*SOL: p=.935; VLR: p=.978; VLW: p=.217*) and HS+EE (*SOL: p=.999; VLR: p=.855; VLW: p=.403*) as well as EE compared to HS+EE (*SOL: p=.853; VLR: p=.894; VLW: p=.833*).

48hr

SOL (***CON***=3; 54718601±5998325; ***EE***=8; 76645228±23895526; ***HS+EE***=8; 73554507± 23794117)
VLR (***CON***=3; 59610097±1531669; ***EE***=8; 56496705±16423238; ***HS+EE***=8; 49270037±4713228)
VLW (***CON***=3; 15130711±2601159; ***EE***=8; 13463864±2716538; HS+EE=8; 13673163±2755272) i

Significance levels for 48hr

CON compared to EE (*SOL: p=.342; VLR: p=.913; VLW: p=.645*) and HS+EE (*SOL: p=.447; VLR: p=.389; VLW: p=.713*)as well as EE compared to HS+EE (*SOL: p=.959; VLR: p=.427; VLW: p=..987*).

Raw data for pERK1/2

2hr

SOL (***CON***=3; 1281073±595130; ***EE***=8; 4453747±2239186; ***HS+EE***=8; 4087533±2500790)
VLR (***CON***=3; 23409048±15076949; ***EE***=8; 15797883±4241690; ***HS+EE***=8; 16519114±9314893)
VLW (***CON***=3; 5591608±2875874; ***EE***=8; 6850754±3450561; ***HS+EE***=8; 6779283±4701113)

Significance levels for 2hr

CON compared to EE (*SOL: p=.121; VLR: p=.413; VLW: p=.824*) and HS+EE (*SOL: p=.183; VLR: p=.481; VLW: p=.900*) as well as EE compared to HS+EE (*SOL: p=.942; VLR: p=.985; VLW: p=.976*).

48hr

SOL (***CON***=3; 913114±186539; ***EE***=8; 5063657±6193931; ***HS+EE***=8; 1957082±1322926)

VLR (***CON***=3; 6049356±3218125; ***EE***=8; 10283924±4653865; ***HS+EE***=8; 6330777±5952736)
VLW (***CON***=3; 4113711±1089898; ***EE***=8; 5730377±2058058; ***HS+EE***=8; 4392380±2591392)

Significance levels for 48hr

CON compared to EE (*SOL: p=.334; VLR: p=.459; VLW: p=.543*) and HS+EE (*SOL: p=.928; VLR: p=.996; VLW: p=.981*) as well as EE compared to HS+EE (*SOL: p=.325; VLR: p=.298; VLW: p=.468*).

Raw data for ratio of p/t ERK1/2.

2hr

SOL (***CON***=3; 0.0258±0.0092; ***EE***=8; 0.0930±0.0441; ***HS+EE***=8; 0.0804±0.0477)
VLR (***CON***=3; 0.6267±0.4831; ***EE***=8; 0.4239±0.1088; ***HS+EE***=8; 0.4887±0.2586)
VLW (***CON***=3; 0.7349±0.2916; ***EE***=8; 0.6673 ±0.4057; ***HS+EE***=8; 0.5981±0.2960)

Significance levels for 2hr

CON compared to EE (*SOL: p=.084; VLR: p=.477; VLW: p=.956*) and HS+EE (*SOL: p=.179; VLR: p=.704; VLW: p=.832*) as well as EE compared to HS+EE (*SOL: p=.831; VLR: p=.866; VLW: p=.917*).

48hr

SOL (***CON***=3; 0.0169±0.0201; ***EE***=8; 0.0612±0.0672; ***HS+EE***=8; 0.0297±0.0252)
VLR (***CON***=3; ±; ***EE***=8; ±; ***HS+EE***=8; 0.3249±0.2057)
VLW (***CON***=3; 0.2704±0.0401; ***EE***=8; 0.4303±0.1547; ***HS+EE***=8; 0.3249 ±0.2056)

Significance levels for 48hr

CON compared to EE (*SOL: p=.376; VLR: p=.533; VLW: p=.373*) and HS+EE (*SOL: p=.917; VLR: p=.903; VLW: p=.886*) as well as EE compared to HS+EE (*SOL: p=.402; VLR: p=.653; VLW: p=.451*).

Raw data for total c-Src

SOL (***CON***=3; 20377220 ±;3977999; ***EE***=8; 17128394±3696279; ***HS+EE***=8; 24300200±17951048)
VLR (***CON***=3; 2992104±513944; ***EE***=8; 2834206±527760; ***HS+EE***=8; 2188772±622517)
VLW (***CON***=3; 5957222±2143974; ***EE***=8; 6594353±4013056; ***HS+EE***=8; 6497261±2068889)

Significance levels for 2hr

CON compared to EE (*SOL: p=.919; VLR: p=.912; VLW: p=.950*) and HS+EE (*SOL: p=.884; VLR: p=.125; VLW: p=.964*) as well as EE compared to HS+EE (*SOL: p=.484; VLR: p=.090; VLW: p=.998*).

48hrs

SOL (***CON***=3; 20531935±6406617; ***EE***=7; 23001243±;10110414; ***HS+EE***=8; 20981690±6136075)
VLR (***CON***=3; 9900282±1459552; ***EE***=6; 7408331±3861995; ***HS+EE***=6; 5831627±997033)
VLW (***CON***=3; 2397282±82306; ***EE***=8; 1705359±296730; ***HS+EE***=8; 2011459±554459)

Significance levels for 48hr

CON compared to EE (SO*L: p=.714; VLR: p=.405; VLW: p=.103*) and HS+EE (SOL: *p=.881; VLR: p=.116; VLW: p=.312*) as well as EE compared to HS+EE (*SOL: p=.924; VLR: p=.571; VLW: p=.665*).

Raw data for pc-Src527

2hr

SOL (***CON***=3; 10299912 ±2003146; ***EE***=8; 8807499±2855305; ***HS+EE***=8; 10439998±1785886)
VLR (***CON***=3; 545397±173578; ***EE***=8; 1466511±738640; ***HS+EE***=8; 1054837±746511)
VLW (***CON***=3; 1580863±334081; ***EE***=8; 4673119±2814126; ***HS+EE***=8; 5147270±3790516)

Significance levels for 2hr

CON compared to EE (*SOL: p=.622; VLR: p=.157; VLW: p=.335*) and HS+EE (*SOL: p=.996; VLR: p=.540; VLW: p=.241*) as well as EE compared to HS+EE (*SOL: p=.366; VLR: p=.481; VLW: p=.951*).

48hr

SOL (***CO***N=3; 0.0169±0.0201; ***EE***=8; 0.0612±0.0672; ***HS+EE***=8; 0.0297±0.0252)
VLR (***CON***=3; 392815±193715; ***EE***=8; 634227±526302; ***HS+EE***=8; 517386±275354)
VLW (***CON***=3; 4292849±104578; ***EE***=8; 3439350±1249639; ***HS+EE***=8; 3774637±1193175)

Significance levels for 48hr

CON compared to EE (*SOL: p=.995; VLR: p=.755; VLW: p=.179*) and HS+EE (*SOL: p=.973; VLR: p=.940; VLW: p=.438*) as well as EE compared to HS+EE (*SOL: p=.920; VLR: p=.884; VLW: p=.720*).

Raw data for p/t c-Src.

2hr

SOL (***CON***=3; 0.5234 ±0.1688; ***EE***=8; 0.5083±0.1008; ***HS+EE***=8; 0.5341±0.1757),
VLR (***CON***=3; 0.0389±0.0154; ***EE***=8; 0.0704±0.0310; ***HS+EE***=8; 0.0844±0.0556)
VLW (***CON***=3; 0.2800±0.0708; ***EE***=8; 1.1161±0.9540; ***HS+EE***=8; 1.0004±0.8577)

Significance levels for 2hr

CON compared to EE (*SOL: p=.996; VLR: p=.705; VLW: p=.575*) and HS+EE (*SOL: p=.1.00; VLR: p=.496; VLW: p=.679*) as well as EE compared to HS+EE (*SOL: p=.997; VLR: p=.902; VLW: p=.975*).

48hr

SOL (***CON***=3; 0.3066±0.1090; ***EE***=7; 0.2219±0.0647; ***HS+EE***=8; 0.2130±0.0812)
VLR (***CON***=3; 0.0389±0.0154; ***EE***=6; 0.0704±0.0310; ***HS+EE***=8; 0.0844±0.0556)
VLW (***CON***=3; 1.7919±0.0652; ***EE***=8; 1.9343±0.4772; ***HS +EE***=8; 1.8891±0.3179)

Significance levels for 48hr

CON compared to EE (*SOL: p=.434; VLR: p=.548; VLW: p=.996*) and HS+EE (*SOL: p=.420; VLR: p=.304; VLW: p=.969*) as well as EE compared to HS+EE (*SOL: p=.999; VLR: p=.832; VLW: p=.918*).